EVOLUTION OF THE SEXUAL REPRODUCTION IN *VERONICA* (PLANTAGINACEAE):

PHYLOGENY, PHYLOGEOGRAPHY AND INVASION

Dissertation zur Erlangung des Grades
Doktor der Naturwissenschaften

Am Fachbereich Biologie
der Johannes Gutenberg-Universität Mainz

Romain Scalone
geboren am 7 Mai 1981 in Colombes
Hauts de Seine (Frankreich)

Mainz, 2011

Dekan:

1 Berichterstatter:

2 Berichterstatter:

Tag der mündlichen Prüfung: 20. Dezember 2011

« Nothing in biology makes sense except in the light of evolution. »

Theodosius DOBZHANSKY

(1900-1975)

« We do not even in the least know the final cause of sexuality;
why new beings should be produced by the union of the two sexual elements, […]
The whole subject is as yet hidden in darkness. »

Charles DARWIN

(1809-1882)

Veronica filiformis Smith

LIST OF FIGURE

LIST OF TABLE

LIST OF APPENDIX

LIST OF ILLUSTRATION

1 INTRODUCTION

1.1 REPRODUCTION IN PLANTS

The reproduction is one of the three fundamental features present in all known living organisms (with nutrition & growth). It is the biological process by which new individual plants (offsprings) are produced from old individual plants (parents) and by which genes are transmitted through generations. The reproductive system exhibits a large diversity with various morphological, physiological and genetic mechanisms among the Angiosperms (or *Magnoliophyta*). These different forms of reproduction can be grouped in two main types: sexual and asexual reproduction. The sexual reproduction creates one new individual genetically different from his parents whereas the asexual one creates one new individual genetically identical to his progenitor. Each of these two reproductive types presents evolutionary advantages which could explain their origins and maintenance along the tree of life. The evolutionary success of Angiosperms is mainly due to the presence of one new sexual organ, the flower, improving the mechanism of sexual reproduction in plants. Within Angiosperms, certain species produce perfect flowers, which correspond to hermaphrodite flowers composed by male and female organs (e.g., stamen and carpel) on the same flower while other species present complete flowers defined as perfect flowers with organs helping to attract pollinators (petals, corolla) and to protect the future flowers or buds (sepals, calyx). Along the evolution of Angiosperms, numerous mechanisms appeared to improve the success of sexual reproduction in two different ways by promoting self-fertilization (cleistogamy, homogamy with facultative or obligate autogamy) or self-sterility (chasmogamy, dichogamy, heterostyly, sporophytic or gametophytic self-incompatibilities, allogamy, facultative or obligate xenogamy).

1.2 LAWS IN SEXUAL REPRODUCTION

These two different ways and their consequences on plant evolution were intensively studied and certain laws were found by botanists after experimental works or field observations. Two examples of such laws will be discussed here: i) Cruden´s law and ii) Baker´s rule. In 1977, Cruden invented the parameter, pollen-ovule ratio (P-O) for the inference of the breeding system. After the estimation of this measure for several species of numerous different plant families, he observed a relationship between the (logarithmic) variation of this measure and the different types of sexual reproduction of species: “The more efficient the transfer of pollen, the lower the P-O should be” (Cruden, 1977). Several others studies were published in the last forty years to confirm the existence of this relationship within all existing families or

genera (Gallardo *et al.*, 1994; Wyatt *et al.*, 2000; Jürgens *et al.*, 2002; Wang *et al.*, 2004; Chouteau *et al.*, 2006[A]; Yang & Guo, 2007), to infer the factors influencing this relationship inter- and intraspecifically (e.g., flowering time, taxonomy, life history, ploidy, phylogeny, environment) (Cruden, 1976; Cruden & Miller-Ward, 1981; Lloyd & Bawa, 1984; Cruden & Lyon, 1985[A], 1985[B]; Lloyd, 1987; Chouteau *et al.*, 2006[A], 2006[B]; Yang & Guo, 2007; Mazer *et al.*, 2007, 2009; Michalski & Durka, 2009) and to understand the relationship in the light of the sex allocation theory (Charlesworth & Charlesworth, 1981; Charnov, 1982; Chouteau *et al.*, 2006[B], 2008). Twenty-three years before, Baker made notes after observations introduced plant species on island (Baker, 1955). During the following years, his note that: "with self-compatible individuals a single propagule is sufficient to start a sexually-reproducing colony (after long-distance dispersal), making its establishment much more likely than if the chance of two self-incompatible yet cross-compatible individuals sufficiently close together spatially and temporally is required" (Baker, 1967; Stebbins, 1957). Baker´s rule can be summarized by the fact that a good invasive species must be a self-compatible species rather a self-incompatible species (Baker, 1974; Rambuda & Johnson, 2004). However, intense discussions took place about the importance of genetic diversity for the invader and its adaptation to a new environment (Hollingworth & Bailey, 2000; Poulin *et al.*, 2005; Grimsby *et al.*, 2007; Novak, 2007; Culley & Hardiman, 2009). Several evolutionary assumptions about the reason(s) of successful and unsuccessful invasions in plants were formulated (e.g., enemy release, general purpose-genotypes, local adaptation, multiple introductions) and tested on model species (Gabrielsen & Brochmann, 1998; Li & Ge, 2001; Bleeker, 2003; Genton *et al.*, 2005; Barney *et al.*, 2006; Tiébré *et al.*, 2007). These two examples of laws about the plant sexual reproductions and their influences on new introductions are subject to discussion. However, these two laws were not studied yet in the case of the *Veronica* genus (Plantaginaceae), although numerous traits of this genus are interesting for their discussions.

1.3 THE VERONICA MODEL

Indeed, the genus *Veronica* (Plantaginaceae sensu APG 2009) offers several traits which are ideal for the study of sexual reproductive systems. The genus *Veronica* is a species-rich genus with approximately 450 species, with about 300 present in the Northern Hemisphere and mainly in Europe and Middle Asia. Two thirds of the European species are located in the Balkan Peninsula, which is one of the major European biodiversity hotspots. The species of *Veronica* are located in a large variety of habitats (e.g., forest, aquatic habitats, dry steppes) and grow from sea levels to the alpine region. The genus *Veronica* shows also a large diversity of life-forms (e.g., therophytes, 1-1.5m tall herbs, subshrubs and shrubs). A certain

number of *Veronica* species are endemics (e.g., Balkan Peninsula), while other are widely cultivated or are cosmopolitan weeds (e.g., *V. persica*, *V. longifolia*, *V. arvensis*, *V. serpyllifolia*). Cosmopolitan weeds or invasive species could be perfect models in order to study the implications of the sexual reproduction in the adaptation to new environments. The phylogenetic relationships within the *Veronica* genus start to be well supported based on molecular data (Albach & Chase, 2001, 2004; Albach *et al.*, 2004[A], 2004[B], 2005; Albach & Meudt, 2010) and are generally congruent with data from chromosome numbers, morphology, life form and phytochemistry (Albach *et al.*, 2004[B]). Little is known about the evolution of the sexual reproductive systems within *Veronica* except from crossing studies conducted in the first half of the 20th (Lehmann, 1909, 1915, 1919, 1922; Härle, 1932; Beatus, 1935; Scheerer, 1937; Schmitz, 1946; Lehmann & Schmitz-Lohner, 1954). However, the genus seems to exhibit a large range of sexual reproductive systems varying from cleistogamous (e.g., *V. peregrina*; Cruden, 1977) to xenogamous (e.g., *V. filiformis*; Lehmann, 1944).

1.4 METHODS, AIMS & ASKS

Therefore, almost all methods of investigation from the molecular level to the entire living plants have been used during the different parts of this thesis to determine the degree of sexual reproduction in *Veronica* species, as for example with DNA-fingerprints by Amplified Fragment-Length Polymorphim (Paper II & III), the DNA sequencing with nuclear, plastid and ribosomal markers (Paper II), the measure of genome size (Paper II), measures of primary and secondary reproductive traits (Paper I), determination of pollen-ovule ratio (Paper I & III), estimation of floral investment added to countings of normal and mutant-flowers (Paper III), of seed investment by counting the seed number and measuring seed and embryo size (Paper III) or of reproductive success by seed viability test using tetrazolium and germination tests (Paper III) and crossing (Paper III) and controlled-environment experiments (Paper I) after transplantation to a greenhouse and pollen tube growth experiments realized with a fluorescence microscope (Short Research Notes),. These methods have been used in order to follow the thread of this dissertation: the study of the evolution of the sexual reproduction(s) in *Veronica* during the course of evolution. Three different scales of time were investigated here:

i) the genus level (*Veronica* genus; Paper I),
ii) the subgenus level (*V.* subg. *Stenocarpon*; Paper II) and
iii) the species level (*V. filiformis*; Paper III).

Thus, the evolution of sexual reproduction has occurred between a range of around seven millions years for the genus level (Paper I) to only one century for the species level (Paper III). Species or populations evolve along time but are also dependent on the parameter space. This is the reason why the evolution of *Veronica* in the Balkan Peninsula (Paper II), in the Caucasian Mountains as well as in the European turfs (Paper I & III) has been studied too. Consequently, this manuscript wants to illustrate the evolution of sexual reproduction(s) in *Veronica* following all different possible biological aspects: three levels of time from genus to species, three regions of speciation from the Balkan Peninsula to the European turfs and several levels of analyses from the complete genome to the population. Several questions have been asked in the three different parts of this thesis:

- How did the sexual reproductive systems evolve in *Veronica*? Can we predict the sexual reproductive system of a *Veronica* species by determining its P-O ratio? Are the measures of the P-O ratio conservative? Which factors can modify it (ploidy, taxonomy, environment)? Do the relationships between the *Veronica* reproductive traits follow Cruden´s law and the sex allocation theory? Which reproductive traitsof *Veronica* do or do not evolve with the sexual reproductive system?
- How did the *V.* subgenus *Stenocarpon* evolve in the Balkan Peninsula? Is the taxonomy of the *V.* subgenus *Stenocarpon* affected by certain interspecific sexual reproductions in this region? Can it be observable on morphology and genetics of species or subspecies? Was the Balkan Peninsula a refugium of *Veronica* diversity during the Pleistocene? Are the conclusions based on the data of nuclear, plastid and ribosomal markers congruent together? Are the conclusions based on the genetic and morphological data congruent too?
- How did the sexual reproductive system of an obligate outcrossing species, *Veronica filiformis*, evolve during an invasion? How can an obligate outcrosser invade all the Europe in only one century without seed production? What are the reasons of this absence of seed in Europe? Are there clones of *V. filiformis* in Europe? Are they good invaders? Were there multiple introductions of *V. filiformis* in Europe? Does *V. filiformis* follow Baker´s rule? How does *V. filiformis* circumvent the law? Which consequence has the deviation of the Baker´s rule on the reproductive system of this species?

Finally, the complete thesis permits the visualization of how the sexual reproduction can evolve within a short period (~100 y.) during certain exceptional situations (Paper III) and how this evolution could participate to have a large diversity of sexual systems within the

genus *Veronica* (Paper I). Discussions are made also on the impact of sexual system on the speciation and the taxonomy (Paper II), the migration into a new habitat (Paper III) as well as on the respect or not of biological laws within *Veronica* (Paper I & III).

1.5 REFERENCES

Albach D.C. and Chase M.W. (2001) Paraphyly of *Veronica* based on sequences from the internal transcribed spacers (ITS) of nuclear ribosomal DNA. *Journal of Plant Research* **114:** 9–18.

Albach D.C. and Chase M.W. (2004) Incongruence in *Veroniceae* (Plantaginaceae): evidence from two plastid and a nuclear ribosomal DNA region. *Molecular Phylogenetics and Evolution* **32:** 183–197.

Albach D.C., Martinez-Ortega M.M., Chase M.W. (2004[A]) *Veronica*: parallel morphological evolution and phylogeography in the Mediterranean. *Plant Systematics and Evolution* **246:** 177–194.

Albach D.C., Martinez-Ortega M.M., Fischer M.A., Chase M.W. (2004[B]) A new classification of the Veroniceae – Problems and possible solution. *Taxon* **53:** 429-452.

Albach D.C., Meudt H.M., Oxelman B. (2005) Piecing together the "new" Plantaginaceae. *American Journal of Botany* **92:** 297-315.

Albach D.C. and Meudt H.M. (2010) Phylogeny of *Veronica* in the Southern and Northern Hemispheres based on plastid, nuclear ribosomal and nuclear low-copy DNA. *Molecular Phylogenetics and Evolution* **54:** 457-471.

Baker H.G. (1955) Self-compatibility and establishment after "long-distance" dispersal. *Evolution* **9:** 347-349.

Baker H.G. (1967) Support for Baker's law-as a rule. *Evolution* **21:** 853-856.

Baker H.G. (1974) The Evolution of weeds. *Annual Review of Ecology and Systematics* **5:** 1-24.

Barney J., Tharayil N., DiTommaso A. and Bhowmik P. (2006) The biology of invasive alien plants in Canada. 5. *Polygonum cuspidatum* Sieb.&Zucc. [= *Fallopia japonica* (Houtt.) Ronse Decr.]. *Canadian Journal of Plant Science* **85:** 887-906.

Beatus R. (1935) Genetische und zytologische Untersuchungen in der *Veronica*-Gruppe *Fruticulosae* sekt. *Veronicastrum*. *Flora* **130:** 153-175.

Bleeker W. (2003) Hybridization and *Rorippa austriaca* (Brassicaceae) invasion in Germany. *Molecular Ecology* **12:** 1831-1841.

Charlesworth D. and Charlesworth B. (1981) Allocations of resources to male and female functions in hermaphrodites. *Biological Journal of the Linnean Society* **15:** 57-74.

Charnov E.L. (1982) The theory of sex allocation. Princeton, NJ, USA.

Chouteau M., Barabé D., Gibernau M. (2006[A]) A comparative study of inflorescence characters and pollen-ovule ratios among the genera *Philodendron* and *Anthurium* (Araceae). *International Journal of Plant Sciences* **167:** 817-829.

Chouteau M., Barabé D., Gibernau M. (2006[B]) Pollen-ovule ratios in some Neotropical Araceae and their putative significance. *Plant Systematics and Evolution* **257:** 147-157.

Chouteau M., Gibernau M., Barabé D. (2008) Relationships between floral characters, pollination mechanisms, life forms, and habitats in *Araceae*. *Botanical Journal of the Linnean Society* **156:** 29-42.

Cruden R.W. (1976) Intraspecific variation in pollen-ovule ratios and nectar secretion: preliminary evidence of ecotypic adaptation. *Annals of the Missouri Botanical Garden* **63:** 277-289.

Cruden R.W. (1977) Pollen-ovule ratios: a conservative indicator of breeding systems in flowering plants. *Evolution* **31:** 32-46.

Cruden R.W. and Lyon D.L. (1985[A]) Patterns of biomass allocation to male and female functions in plants with different mating systems. *Oecologia* **66:** 299-306.

Cruden R.W. and Lyon D.L. (1985[B]) Correlations among stigma depth, style length, and pollen grain size: do they reflect function or phylogeny. *Botanical Gazette* **146:** 143-149.

Cruden R.W. and Miller-Ward S. (1981) Pollen-ovule ratio, pollen size, and the ratio of stigmatic area to the pollen-bearing area of the pollinator: an hypothesis. *Evolution* **35:** 964-974.

Culley T. and Hardiman N. (2009) The role of intraspecific hybridization in the evolution of invasiveness: a case study of the ornamental pear tree *Pyrus calleryana*. *Biological Invasions* **11:** 1107-1119.

Gabrielsen T. and Brochmann C. (1998) Sex after all: high levels of diversity in the artic clonal plant *Saxifraga cernua* using RAPD markers. *Molecular Ecology* **7:** 1701-1708.

Gallardo R., Dominguez E., Munoz J.M. (1994) Pollen-ovule ratio, pollen size, and breeding system in *Astragalus* (Fabaceae) subgenus *Epiglottis*: a pollen and seed allocation approach. *American Journal of Botany* **81:** 1611-1619.

Genton B., Shykoff J., Giraud T. (2005) High genetic diversity in French invasive populations of common ragweed, *Ambrosia artemisiifolia*, as a result of multiple sources of introduction. *Molecular Ecology* **14:** 4275-4285.

Grimsby J., Tsirelson D., Gammon M., Kesseli R. (2007) Genetic diversity and clonal versus sexual reproduction in *Fallopia spp.* (Polygonaceae). *American Journal of Botany* **94:** 957-964.

Härle A. (1932) Die Arten und Formen der *Veronica*-Sektion *Pseudolysimachia* Koch auf Grund systematischer und experimenteller Untersuchungen. *Bibliotheca Botanica* **26:** 1-86.

Hollingsworth M. and Bailey J. (2000) Hybridisation and clonal diversity in some introduced *Fallopia* species. *Watsonia* **23:** 111-121.

Jürgens A., Witt T., Gottsberger G. (2002) Pollen grain numbers, ovule numbers and pollen-ovule ratios in Caryophylloideae: correlation with breeding system, pollination, life form, style number, and sexual system. *Sex Plant Reproduction* **14:** 279-289.

Lehmann E. (1909) Über Zwischenrassen in der *Veronica*-Gruppe *Agrestis. Zeitschrift für induktive Abstammungs- und Vererbungslehre* **2:** 145-208.

Lehmann E. (1915) Lichtkeimungsfragen. *Zeitschrift für Botanik* **7:** 560-580.

Lehmann E. (1919) Über die Selbststerilität von *Veronica syriaca. Zeitschrift für induktive Abstammungs- und Vererbungslehre* **21:** 1-47.

Lehmann E. (1922) Über die Selbststerilität von *Veronica syriaca* II. *Zeitschrift für induktive Abstammungs- und Vererbungslehre* **27:** 161-177.

Lehmann E. (1944) *Veronica filiformis* Sm., eine selbsterile Pflanze. *Jahrbücher für wissenschaftliche Botanik* **91:** 395-403.

Lehmann E. and Schmitz-Lohner M. (1954). Entwicklung und Polyploidie in der *Veronica*-Gruppe *Agrestis. Zeitschrift für induktive Abstammungs- und Vererbungslehre* **86:** 1-34.

Li A. and Ge S. (2001) Genetic variation and clonal diversity of *Psammochloa villosa* (Poaceae) detected by ISSR markers. *Annals of Botany* **87:** 585-590.

Lloyd D.G. (1987) Allocations to pollen, seeds and pollination mechanisms in self-fertilizing plants. *Functional Ecology* **1**: 83-89.

Lloyd D.G. and Bawa K.S. (1984) Modification of the gender of seed plants in varying conditions. *Evolutionary Biology* **17:** 255-338.

Mazer S.J., Delesalle V.A., Paz H. (2007) Evolution of mating system and the genetic covariance between male and female investment in *Clarkia* (Onagraceae): selfing opposes the evolution of trade-offs. *Evolution* **61:** 83-98

Mazer S.J., Dudley L.S., Delesalle V.A., Paz H., Galusky P. (2009) Stability of pollen-ovule ratios in pollinator-dependent versus autogamous *Clarkia* sister taxa: testing evolutionary predictions. *New Phytologist* **183:** 630-648.

Michalski S.G. and Durka W. (2009) Pollination mode and life form strongly affect the relation between mating system and pollen to ovule ratios. *New Phytologist* **183:** 470-479.

Novak S. (2007) The role of evolution in the invasion process. *Proceedings of the National Academy of Sciences of the United States of America* **104:** 3671-3672.

Poulin J., Weller S., Sakai A. (2005) Genetic diversity does not affect the invasiveness of fountain grass (*Pennisetum setaceum*) in Arizona, California and Hawaii. *Diversity and Distributions* **11:** 241-247.

Rambuda T. and Johnson S. (2004) Breeding systems of invasive alien plants in South Africa: does Baker's rule apply? *Diversity and Distributions* **10:** 409-416.

Scheerer H. (1937) Experimentelle und zytologische Untersuchungen innerhalb der *Veronica*-Gruppe *Pentasepala*. *Flora* **131:** 287-323.

Schmitz M. (1946) Zur Charakterisierung der Arten der *Veronica*-Gruppe *Agrestis* unter besonderer Berücksichtigung des Polyploidieproblems. Tübingen, University Tübingen.

Stebbins L. (1957) Self fertilization and population variability in the higher plants. The *American Naturalist* **91:** 337-354.

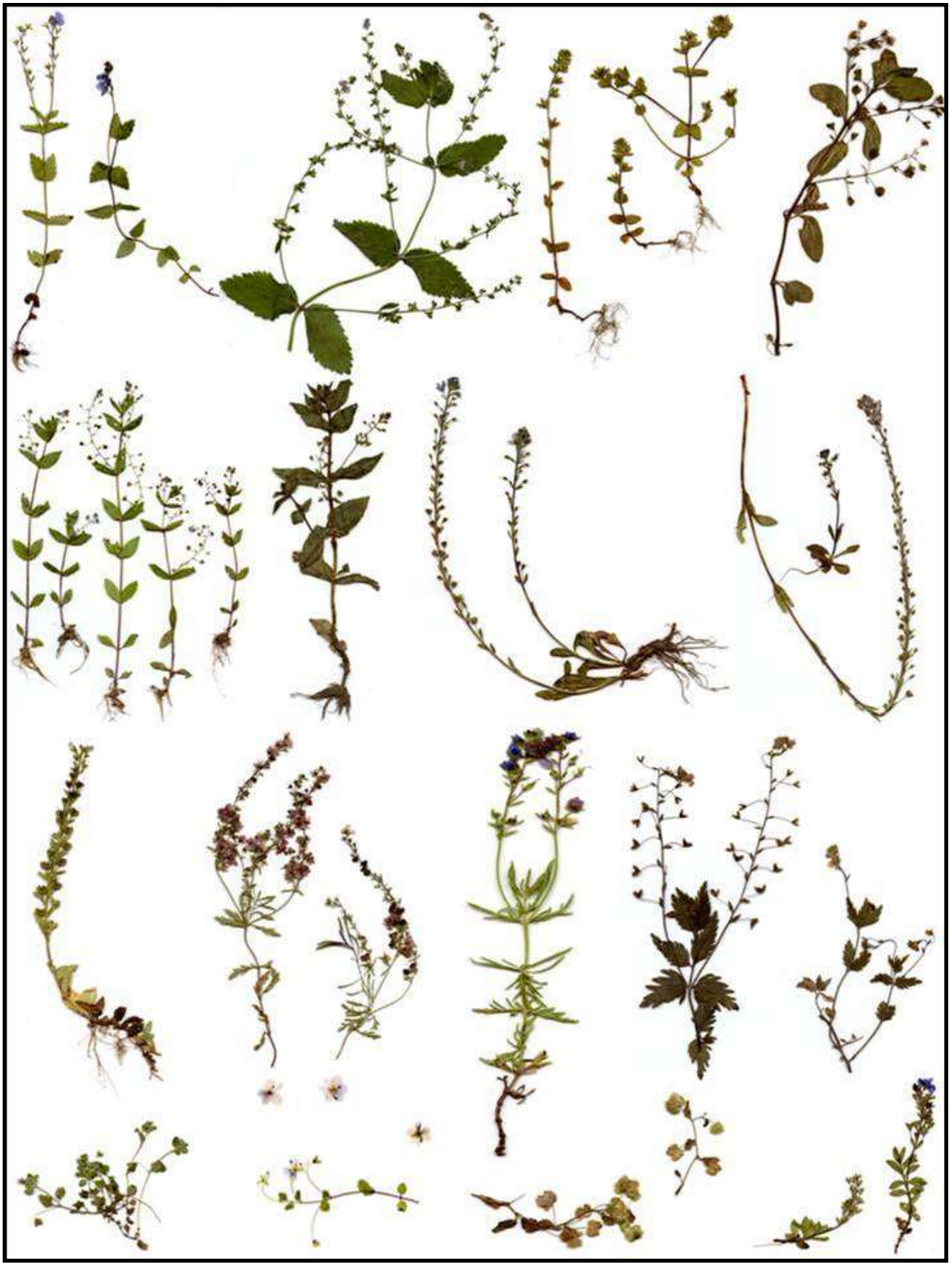
Tiébré M.-S., Bizoux J.-P., Hardy O., Bailey J., Mahy G. (2007) Hybridization and morphogenetic variation in the invasive alien *Fallopia* (Polygonaceae) complex in Belgium. *American Journal of Botany* **94:** 1900-1910.

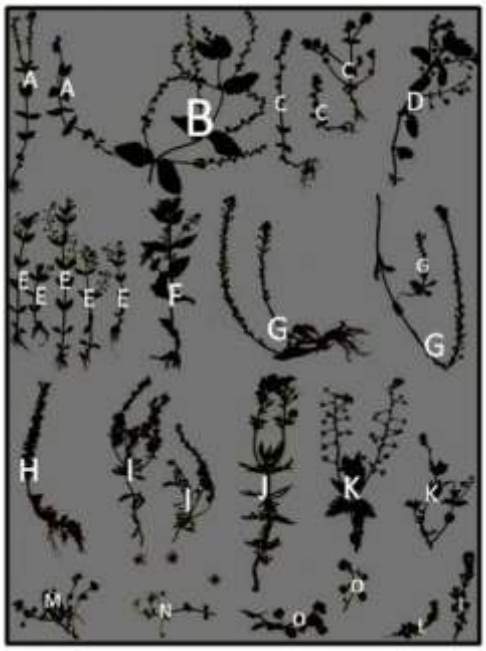
Wang Y.-Q., Zhang D.-X., Chen Z.-Y. (2004) Pollen histochemistry and pollen : ovule ratios in Zingiberaceae. *Annals of Botany* **94:** 583-591.

Wyatt R., Broyles S.B., Lipow S.R. (2000) Pollen-Ovule ratios in Milkweeds (Asclepiadaceae): an exception that probes the rule. *Systematic Botany* **25:** 171-180.

Yang C.-F. and Guo Y.-H. (2007) Pollen-ovule ratio and gamete investment in *Pedicularis* (Orobranchaceae). *Journal of Integrative Plant Biology* **49:** 238-245.

“DIVERSITY OF LIFE-FORM IN *VERONICA*”





A *Veronica chamaedrys* (*V.* subg. *Chamaedrys*)

B *Veronica magna* (*V.* subg. *Chamaedrys*)

C *Veronica arvensis* (*V.* subg. *Chamaedrys*)

D *Veronica beccabunga* (*V.* subg. *Beccabunga*)

E *Veronica anagalloides* (*V.* subg. *Beccabunga*)

F *Veronica anagallis-aquatica* (*V.* subg. *Beccabunga*)

G *Veronica gentianoides* (*V.* subg. *Beccabunga*)

H *Veronica serpyllifolia* (*V.* subg. *Beccabunga*)

I *Veronica orientalis* (*V.* subg. *Pentasepalae*)

J *Veronica multifida* (*V.* subg. *Pentasepalae*)

K *Veronica caucasica* (*V.* subg. *Pentasepalae*)

L *Veronica vendettae* (*V.* subg. *Pentasepalae*)

M *Veronica polita* (*V.* subg. *Pocilla*)

N *Veronica filiformis* (*V.* subg. *Pocilla*)

O *Veronica crista-galli* (*V.* subg. *Cochlidiosperma*)

2 PHYLOGENY OF SEXUAL REPRODUCTION

"Evolution of the pollen-ovule ratio in* Veronica *(Plantaginaceae)"

by R. Scalone[1], M. Kolf[1] and D.C. Albach[2, §]

from:

[1] Institut für Spezielle Botanik und Botanischer Garten, Johannes Gutenberg Universität, Mainz, Germany

[2] Institut für Biologie und Umweltwissenchaften (IBU), Carl von Ossietzky Universität, Oldenburg, Germany

[§] author for correspondence: Prof. Dr. D. C. Albach, Institut für Biologie und Umweltwissenchaften (IBU), Carl von Ossietzky Universität, Carl von Ossietzky-str. 9-11, 26111 Oldenburg, Germany.

Email: dirk.albach@uni-oldenburg.de,

Phone: (0049) - 441 798 3339,

Fax: (0049) - 441 798 3331,

Keywords: Character correlation; Flower morphology; Interspecific variation; Pollen-ovule ratio; Reproductive traits; *Veronica*.

Running head: Pollen-ovule ratios in *Veronica*

P-O = pollen-ovule ratio ; RT = reproductive traits ; Sta-Sty = stamen-style ratio.

2.1 INTRODUCTION

The pollen-ovule ratio (P-O) is a commonly used character to infer the type of sexual reproduction in flowering plants (Gallardo *et al.*, 1994; Wyatt *et al.*, 2000; Jürgens *et al.*, 2002; Wang *et al.*, 2004; Chouteau *et al.*, 2006[A]; Yang & Guo, 2007) or to indicate the distribution of sexual resources within plants (Wyatt, 1984; Chouteau *et al.*, 2006[B], 2008). Resources are limited in a plant according to the sex allocation theory and the amount allocated to sexual reproduction should be distributed effectively between male and female functions in hermaphrodite flowers (Charlesworth & Charlesworth, 1981; Charnov, 1982). Two clear opposite trends exist in such flowers: selfing species are defined by their ability to successfully self-fertilize (cleistogamy, homogamy with facultative or obligate autogamy) in contrast to outcrossing species, which possess morphological, physiological and/or genetic mechanisms to prevent selfing (chasmogamy, dichogamy, heterostyly, self-incompatibility, allogamy with facultative or obligate xenogamy). Cruden (1977) argued that the P-O approximates these trends observed in plants by estimating the pollination efficiency and reflecting the predictability of pollinators in a habitat. Thus, the P-O can be interpreted as the

likelihood of sufficient pollen grains reaching each stigma to result in maximum seed set (Cruden, 1977). On the basis of this interpretation, the relationship of the P-O to the sexual reproductive system has repeatedly been re-evaluated (Wyatt *et al.,* 2000; Chouteau *et al.,* 2006[A], 2006[B]; Götzenberger *et al.,* 2006, 2007; Mazer *et al.,* 2007, 2009). Although self-fertilizing species allocate relatively less to male function than do outcrossing species (Cruden, 1977; Jürgens *et al.,* 2002) and therefore have a lower P-O, many other factors affect male function and can influence the P-O, such as environment (Lloyd & Bawa, 1984; Lloyd, 1987), flowering time (Mazer *et al.,* 2007, 2009), life history (Cruden & Lyon, 1985[B]; Yang & Guo, 2007; Michalski & Durka, 2009) and phylogeny (Chouteau *et al.,* 2006[A], 2006[B]). Whereas the significance of the P-O is reasonably well-studied between species (Gallardo *et al.,* 1994; Wang *et al.,* 2004) and across the angiosperms (Cruden, 1976, 1977), the variation between populations within one species has rarely been examined systematically (Mazer *et al.,* 2007, 2009; Delesalle *et al.,* 2008). Studies focusing on both intra- and interspecific variation within one genus are rare.

The genus *Veronica* (Plantaginaceae sensu APG 2009) offers several advantages for such a study: First, *Veronica* is a species-rich genus with approximately 400 species. Second, its species are found in a variety of habitats (e.g., alpine regions, aquatic habitats, dry steppes) and have a large diversity of life-forms (e.g., therophytes, 1-1.5m tall herbs, subshrubs and shrubs). Third, a certain number of *Veronica* species are widely cultivated or are cosmopolitan weeds (e.g., *V. persica*, *V. longifolia*, *V. arvensis*, *V. serpyllifolia*) and could be excellent models for testing intraspecific variation of P-O. Fourth, a well-supported phylogenetic hypothesis of *Veronica* exists based on molecular data (Albach & Chase, 2001, 2004; Albach *et al.,* 2004[A], 2004[B], 2005; Albach & Meudt, 2010). Based on this information, *Veronica* has been subdivided into thirteen subgenera, which are generally congruent with data from chromosome numbers, morphology, life form and phytochemistry (Albach *et al.,* 2004[B]). However, little is known about the evolution of the sexual reproductive systems within *Veronica* except from crossing studies conducted in the first half of the 20th century (Lehmann, 1909, 1915, 1919, 1922; Beatus, 1935; Härle, 1932; Scheerer, 1937; Schmitz, 1946; Lehmann & Schmitz-Lohner, 1954). The genus appears to exhibit a large range of sexual reproductive systems varying from cleistogamous (e.g., *V. peregrina;* Cruden, 1977) to xenogamous (e.g., *V. filiformis*; Lehmann, 1944). However, the form of the flower has mostly remained stable with four blue - lilac to pink or white petal lobes, two stamens and one style, making the genus an ideal system in which to examine variation in allocation to various traits associated with sexual reproduction.

For these reasons, the objectives of this study were (1) to investigate the P-O of 46 *Veronica* species from nine of the eleven subgenera from the Northern Hemisphere in order to estimate their sexual reproductive systems, (2) to compare these predictions with crossing data from the literature, (3) to examine the inter- and intraspecific variability of the P-O within *Veronica* in relation to several intrinsic and extrinsic factors (taxonomy, ploidy, environment) and finally (4) to discuss the impact of plastic response of P-O variation in reaction to varying quantities of light in a controlled-environment experiment with *V. persica.* These results on inter- and intraspecific variation of P-O within *Veronica* allow us to draw important conclusions for the general discussion of P-O and its relationships with other reproductive traits (RT) in the light of sex allocation theory.

2.2 MATERIALS AND METHODS

2.2.1 Plant material

A total of 68 accessions from different localities of 45 *Veronica* species was investigated (Table 1), some cultivated in the botanical garden of the Mainz University (Germany) (n = 33), some collected from wild plants in their native habitats (n = 18), and some from herbarium specimens (n = 17). Intraspecific variability of the P-O was investigated by studying multiple accessions for thirteen species and in a controlled-environment experiment on the weedy and cosmopolitan species, *Veronica persica*. Three species (*V. arvensis*, *V. serpyllifolia* and *V. peregrina*) had been studied before by Cruden (1977) and the P-O of the first two were compared to our results (Table 2). Cruden´s results for *V. peregrina* (Cruden, 1977) have been included in our analysis as we did not investigate that species ourselves (Table 1).

2.2.2 Pollen-ovule ratio

Mature buds were collected in the field or in the botanical garden immediately prior to anthesis and preserved in 0.9 ml of 70% ethanol. Each mature bud came from one separate individual. Closed mature buds collected from herbarium specimens were soaked in 0.9 ml 70% ethanol for one week in order to facilitate dissection and counting of ovules and pollen grains. To count pollen grain number, the two anthers of one flower were dissected carefully under the binocular microscope. Two drops of methylenblue MTT (3 g of methylene blue in 1 l of 20% ethanol) and glycerine were added to the suspension for a total of 1 ml and stirred together for 30 sec with a vortex mixer. Then, 1 µl was transferred onto a slide and pollen grains were counted with the aid of a microscope. This was repeated ten times per flower. The

total number of pollen grains was calculated as the mean of the counted pollen grains in the ten drops multiplied by 1000 (factor of dilution). Ovaries were carefully dissected and the number of ovules per flower was counted under the binocular microscope. The P-O was scored as the total number of pollen grains in the two anthers divided by the number of ovules in the ovary of one flower.

2.2.3 Intraspecific variation of P-O: controlled-environment experiment

To determine if the environment can significantly modify P-O, the influence of light on pollen and ovule production was tested in *V. persica* in the greenhouse of the botanical garden of Mainz between September 2007 and February 2008. Light is an ecologically important parameter that differs in various habitats and seasons and is easy to control in a greenhouse. The weedy *Veronica persica* was selected because of its proven phenotypic plasticity and the easy access to the large quantity of seeds required for such an experiment. Fifty individuals were exposed to normal light levels in the greenhouse (not estimated) plus an additional light of 400 Watts at a distance of 90 cm (Philips Son-T400 Sodium lamp) during a period of twelve hours (between 8.00am and 8.00pm) every day and at a temperature of 18-20°C. As a negative control, 47 individuals were grown in the same greenhouse without an additional light source but with the same temperature (NB: Three individuals of the control died during the experiment). All seeds came from the same source (information available from the authors upon request), grew in the same conditions before seedling stage and were randomly assigned to one of the two treatments. The same soil was used for both groups and checked for various growth parameters (information available from the authors upon request). One-sample Kolmogorov-Smirnov tests were conducted on pollen and ovule production and P-O by using SPSS v 15.0 software (SPSS Inc., Chicago, IL, USA): pollen numbers and P-O in this experiment follow a normal distribution ($p > 0.05$) whereas ovule numbers did not ($p < 0.05$). Thus, the non-parametrical Mann-Whitney U-test was used in SPSS to detect differences in ovule production between the two conditions whereas a parametrical T-test was required for the analysis of P-O and pollen number.

2.2.4 Intraspecific variation of P-O: differences between accessions

To test if different biotic or abiotic factors can be responsible for intraspecific variation of the P-O, we sampled multiple accessions of thirteen species (Table 2). Differences between accessions include year of collection (*V. officinalis*), corolla color (*V. schmidtiana*; W for white and B for blue corolla) and collection locality (Table 2). The P-Os of seven species (*V.*

chamaedrys, V. serpyllifolia, V. orientalis, V. persica, V. filiformis, V. polita and *V. caucasica*) were determined from accessions cultivated in the botanical garden of Mainz and directly from their original environment in the Caucasian mountains (Georgia). Differences were tested statistically on the first six above-mentioned species by a nested analysis of variance (nested ANOVA) in order to observe the impact of habitat as a source of variation in pollen and ovule production and P-O measurements, regardless of the species. For the nested ANOVA test, pollen and ovule numbers were square-root transformed and the P-O was log-transformed (Table 3_B).

2.2.5 Interspecific variation of P-O: selfing / outcrossing compared to SC / SI

We inferred the sexual reproductive system of the species based on the log $_{P\text{-}O}$ following the categories defined by Cruden (1977) (Figure 1). To compare statistically our inferences (Table 1) with those of previous crossing studies (Appendix 1), a Pearson product-moment correlation was calculated using SPSS. Data from our 46 species were coded using as either selfing [cleistogamous, obligately or facultatively autogamous sensu Cruden (1977); value 0] or outcrossing [facultatively or obligately xenogamous sensu Cruden (1977); value 1]. The 27 *Veronica* species for which crossing data were available in the literature (Lehmann, 1909, 1915, 1919, 1922; Beatus, 1935; Härle, 1932; Scheerer, 1937; Schmitz, 1946; Lehmann & Schmitz-Lohner, 1954) were classified as self-compatible (SC; value 0) or self-incompatible (SI; value 1). Differences were also sought between primary and secondary reproductive traits (RT) within these two datasets (selfing / outcrossing and SC / SI) to reinforce this correlation between the two datasets (Table 4).

2.2.6 Primary and secondary reproductive characters

To estimate the reproductive investment in each species, several floral characters were measured in our sample of 46 species, although not all characters could be measured in all species (Table 1). The characters, corolla size ($n_{species}$ = 44), style ($n_{species}$ = 43) and stamen lengths ($n_{species}$ = 41) were measured with a graduated microscope. Based on the latter two, the stamen-style ratio (Sta-Sty; $n_{species}$ = 41) was estimated. Data on pollen grain size ($n_{species}$ = 34) and seed size ($n_{species}$ = 44) were collected from the literature (Hong, 1984; Manukian, 1984; Martínez-Ortega *et al.*, 2000; Martínez-Ortega & Rico, 2001; Sánchez Agudo *et al.*, 2009; Table 1 and Appendix 1). One-sample Kolmogorov-Smirnov tests were conducted on the six primary (pollen number, ovule number, P-O, pollen width and length and seed size) and four secondary reproductive characters (corolla size, style and stamen length, Sta-Sty)

within *Veronica* using SPSS: pollen number, P-O, corolla size and style and stamen length follow a normal distribution ($p > 0.05$) whereas ovule number and Sta-Sty did not ($p < 0.05$). Thus, the non-parametrical Mann-Whitney U-test was used in SPSS to detect differences in ovule production and Sta-Sty whereas a parametrical T-test was used for the analysis of the other data (Table 1 & Appendix 1). Differences between the sexual reproductive systems (selfing / outcrossing), between life histories (annual / perennial) and between sexual systems within life histories were analyzed by non parametrical Mann-Whitney-U-test or parametrical T-test depending to the distribution of the datasets (Table 5_A & 5_B).

2.2.7 Relationships between traits and Veronica phylogeny

To examine the evolution of the characters and their relationships, Pearson product-moment correlations were estimated with SPSS (Figure 2). Further, a Bayesian analysis of character evolution and correlations was conducted based on phylogenetic hypothesis for *Veronica* derived from Albach *et al.,* 2004[B] and Albach & Meudt, 2010 (Figure 1) in BayesTraits v1.0 (Pagel *et al.,* 2004). We used the Markov chain Monte Carlo method in the subprogram "Continuous" with a random walk model, 30 million generations, a burnin of 10% and rate deviation controlled to achieve an acceptance of 20-40%. Convergence was checked in Tracer v. 1.5 (Rambaut & Drummond, 2007).

2.3 RESULTS

2.3.1 Primary floral traits

The number of pollen grains per flower ranged from 300 in *V. hederifolia* (*V.* subg. *Cochlidiosperma*) to 55.978 per flower in *V. longifolia* (*V.* subg. *Pseudolysimachium*) (Table 1). The size of pollen grain varied from length x width of 16.1 x 13.8 µm in *V. incana* (*V.* subg. *Pseudolysimachium*) to 37.4 x 35.1 µm in *V. cymbalaria* (*V.* subg. *Cochlidiosperma)* within the 34 investigated species (Appendix 1). The number of ovule ranged from the fixed number of four found in all species of *V.* subg. *Cochlidiosperma* (*V. hederifolia*, *V. sublobata*, *V. cymbalaria*, *V. lycica*) to 89 in *V. longifolia* (*V.* subg. *Pseudolysimachium*) (Table 1). The smallest seeds are the seeds of *V. beccabunga* and *V. scardica* (both *V.* subgenus *Beccabunga*) with a maximum length of only 0.5 mm while the largest are found in *V.* subg. *Cochlidiosperma* (2.8 mm; *V. cymbalaria* and *V. hederifolia*) among the included 44 *Veronica* species (Table 1).

2.3.2 Pollen-ovule ratio

The maximum average P-O is that of *V. lycica* (*V.* subg. *Cochlidiosperma*) with 2.572 and the minimum that of *V. magna* (P-O = 19; *V.* subg. *Chamaedrys*) apart from the literature record of *V. peregrina* (*V.* subg. *Beccabunga*; P-O = 7; Cruden, 1977). Applying Cruden´s classification based on $\log_{P\text{-}O}$ to divide plants in five sexual categories, we identified only one cleistogamous species, *V. peregrina* ($\log_{P\text{-}O} < 1.00$) and one obligate xenogamous species, *V. lycica* ($\log_{P\text{-}O} > 3.30$) in addition to nine obligate autogamous ($1.00 \leq \log_{P\text{-}O} \geq 1.80$), thirteen facultative autogamous ($1.80 \leq \log_{P\text{-}O} \geq 2.50$) and twenty-two facultative xenogamous ($2.50 \leq \log_{P\text{-}O} \geq 3.30$) species in our dataset (Figure 1). Subgenus *Beccabunga* represented by nine *Veronica* species (*V. peregrina* included) has the lowest mean $\log_{P\text{-}O}$ with 2.11 ($\mu_{P\text{-}O} = 129$) whereas subgenus *Pentasepalae* ($n_{species} = 8$) has the highest mean $\log_{P\text{-}O}$ with 2.91 ($\mu_{P\text{-}O} = 818$). The $\mu_{P\text{-}O}$ of our 46 *Veronica* species is 315.7 ($\log_{P\text{-}O} = 2.50$; Figure 1).

2.3.3 Intraspecific variation of P-O: controlled-environment experiment

The flowers of *V. persica* from the light-treatment produced more pollen ($n_{pollen} = 6956 \pm 2470$) and more ovules ($n_{ovule} = 21 \pm 3$) than the flowers from the control treatment ($n_{pollen} = 5581 \pm 2262$ / $n_{ovule} = 15 \pm 1$; Table 3_A) and these differences are highly significant (T-test for pollen production: $p = 0.005$ / Mann-Whitney-U-test for ovule production: $p < 0.0001$). However, the flowers that received extra light had a lower P-O (329 ±121) than the controls (383 ±157; Table 3_A) although these values were not significantly different ($p = 0.059$).

2.3.4 Intraspecific variation of P-O: difference between accessions

Flowers of *V. schmidtiana* presenting two different color morphs (W for white and B for blue) from the same population have the same P-O ($P\text{-}O_W = 233.3$, $P\text{-}O_B = 232.5$; Table 2). Similarly flowers of *V. officinalis* do not exhibit any evident difference in P-O between years ($P\text{-}O_{2007} = 225.0$, $P\text{-}O_{2008} = 233.3$; Table 2). The P-Os of specimens collected in populations from the field seem to be higher than the ones determined from herbarium specimens (*V. anagallis-aquatica, V. gentianoides, V. syriaca*; Table 2), while the P-O determined from individuals collected in the Mainz botanical garden were lower than those from herbarium specimen (*V. hederifolia, V. cymbalaria 4N*; Table 2). No statistical tests were performed on these data since only very few populations were available. However, a nested analysis of variance (nested ANOVA) of pollen, ovule and P-O data from paired accessions from the botanical garden and the original habitat of six different *Veronica* species demonstrated large and significant differences between them (Table 3_B). All three primary reproductive traits

exhibit highly significant differences between the two types of localities (origin: botanical garden / field) and among the six species (Table 3_B). Values for all three traits are significantly higher when they are estimated from specimens collected in the field than from specimens collected in the botanical garden. However, only 20% of the variation in pollen number and P-O measurements is associated with the accession´s origin whereas 90% of the variation in ovule number is due to differences among species (Table 3_B).

2.3.5 Interspecific variation of P-O: selfing / outcrossing compared to SC / SI

Seven species were considered self-incompatible and twenty self-compatible (SI / SC: 25.9% / 74.1%; Appendix 1) based on our literature survey. According to these reports the maximum P-O of these self-compatible species is in *V. montana* (*V.* subg. *Veronica*; P-O = 495.0), whereas the minimum P-O of the self-incompatible species is present in *V. teucrium* (*V.* subg. *Pentasepalae*; P-O = 385.9). Based on our P-O data and Cruden´s classification, we inferred twenty-three outcrossing and twenty-three selfing species (Table 1). Despite the rather tentative conclusion, a strong correlation exists between our simplified conclusions based on log $_{P\text{-}O}$ (selfing / outcrossing) and the ones based on the literature (SC / SI) for the twenty-eight *Veronica* species present in both datasets ($r = 0.848$; $p < 0.0001$). Moreover, the same conclusions are obtained when all pairwise comparisons are conducted with primary and secondary reproductive traits (RT) either our inferences based on P-O or those based on previous crossing studies (Table 4).

2.3.6 Secondary floral traits

Based on our survey of secondary reproductive traits, *Veronica donii* (*V.* subg. *Pellidosperma*) has the largest corolla (24 mm) and the largest stamen (8.5 mm) (Table 1), whereas the longest style is present in *V. cusickii* (9.0 mm; *V.* subg. *Veronica;* Table 1). The smallest corollas are those of *V. arvensis* and *V. peregrina* (2.0 mm; Table 1). The former also has the smallest stamens (0.3 mm; *V.* subg. *Chamaedrys*) whereas the latter has the shortest style (0.1 mm; *V.* subg. *Beccabunga*) and the largest stamen-style ratio (Sta-Sty = 10; Table 1). *Veronica glauca* (*V.* subg. *Pellidosperma*) has the lowest Sta-Sty (0.20).

2.3.7 Differences between life histories or reproductive systems

All of the species in our study could be clearly classified as either annuals or perennials except for *V. scardica*, which seems to be a facultative annual (personal observation). Of the remaining, 29 species (64.4%) are perennial, while 16 are annual species (35.6%; Appendix

1). The perennial species have more pollen and ovules, longer styles and stamen and higher P-O than the annual species (all with $p < 0.05$; Table 5_A & 5_B). The annuals have bigger seeds than perennials ($p = 0.0040$; Table 5_A). There is no difference for corolla and pollen size (width & length) and Sta-Sty between *Veronica* annuals and perennials (all with $p > 0.05$; Table 5_A & 5_B). Within the primary RT, P-O and pollen number are higher in outcrossing than in selfing species ($p < 0.0001$ & $p < 0.0001$) while no difference can be observed in seed or pollen size (all with $p > 0.05$; Table 5_B). These differences between outcrossing and selfing species are also found when both annuals and perennials are analyzed separately. In addition, significant differences in ovule number and seed size are observed between the outcrossing and the selfing perennials ($p = 0.0140$ & $p = 0.0340$; Table 5_A). Among the secondary RT, corolla size and style and stamen length are larger and longer, respectively in the outcrossing than in the selfing species ($p = 0.0310$; $p < 0.0001$; $p = 0.006$) except Sta-Sty ($p > 0.05$; Table 5_B). The trends observed in the secondary RT disappeared completely when annuals or perennials were analyzed separately (Table 5_B).

2.3.8 Relationships between reproductive traits

Relations between primary and secondary RT and sexual systems are represented in Figure 2. Pollen and ovule numbers are necessarily positively and negatively related to P-O ($r = 0.501$, $p = 0.0002$; $r = - 0.294$, $p = 0.0235$). The relationship between pollen number and ovule number is also highly significant ($r = 0.497$, $p < 0.0001$). A strong trade-off appears to exist between pollen number and pollen size (length: $r = - 0.620$, $p < 0.0001$ / width: $r = - 0.470$, $p < 0.0001$). A smaller, but still marginally significant negative relationship also exists between ovule number and seed size ($r = - 0.265$, $p = 0.0391$). Pollen size and seed size are strongly correlated (length: $r = 0.621$, $p < 0.0001$ / width: $r = 0.592$, $p < 0.0001$), while no significant relationship exists between pollen size and P-O (length: $r = - 0.062$, $p = 0.3610$ / width: $r = - 0.083$, $p = 0.3175$, data not shown). The P-O is correlated with both, the sexual reproductive system ($r = 0.325$, $p = 0.0286$) and corolla size ($r = 0.429$, $p = 0.0016$). Among secondary RT, stamen, style and corolla sizes are positively and highly significantly correlated together (corolla / stamen: $r = 0.507$, $p = 0.0003$; corolla / style: $r = 0.609$, $p < 0.0001$; stamen / style: $r = 0.890$, $p < 0.0001$) explaining the strong correlations between the stamen - style ratio (Sta-Sty) and both P-O ($r = - 0.399$, $p = 0.004$) and corolla size ($r = - 0.486$, $p = 0.005$). Although seed size and style length were significantly correlated ($r = 0.483$, $p = 0.0021$), pollen size and style length were not (length: $r = - 0.089$, $p > 0.05$ / width: $r = - 0.138$, $p > 0.05$). In addition, a significant correlation exists between the male parts of the flower, pollen number and stamen

length ($r = 0.510$, $p < 0.05$) but not between the female parts of the flower, ovule number and style length ($r = -0.059$, $p > 0.05$).

2.3.9 Reproductive evolution and phylogeny

To further investigate the evolution of sexual RT and the phylogenetic component in the reproductive system, the lambda parameter (λ) was measured for all traits in BayesTraits (Figure 2): λ varies between 0 and 1, with 0 indicating no correlation between the trait and the phylogeny and 1 indicating a perfect correlation. The highest values of lambda are observed for seed size (λ = 0.908), ovule number (λ = 0.902) and life history (λ = 0.925) and the lowest for style length (λ = 0.258), Sta-Sty (λ = 0.296) and pollen size (length and width: λ = 0.296). Within the relationships between the floral traits discussed above, the highest correlation with phylogeny is to be found for the correlation between ovule number and seed size (λ = 0.914) and between ovule number and life history (λ = 0.970, data not shown), while the lowest correlations are observed for the relationship between corolla size and Sta-Sty (λ = 0.190, data not shown) and between the stamen and style lengths (λ = 0.221, data not shown).

2.4 DISCUSSION

2.4.1 Variation of P-O within species of Veronica

Estimation of sexual reproductive systems based on P-O is often complicated by the lack of information on intraspecific variation. Indeed, P-O can be influenced by several factors apart from breeding system: taxonomy, ploidy and environment have all been observed to have an influence on P-O in other genera (Cruden & Kinsman, 1976; Lloyd & Bawa, 1984; Cruden & Lyon, 1985[B]; Lloyd, 1987; Mazer *et al.,* 2007, 2009; Yang & Guo, 2007; Chouteau *et al.,* 2008).

Taxonomy

Cruden (1976) demonstrated an up to ten-fold variation of P-O is possible within a given species. In *Caesalpinia pulcherrima*, for example, P-O varied between 827 ($\log_{P\text{-}O} = 2.92$) and 9416 ($\log_{P\text{-}O} = 3.97$) from one population to another (Cruden, 1976), causing the populations to be classified in different categories of sexual reproductive system, according to Cruden´s classification (1977). This classification in two different categories was also observed in four of our thirteen species of *Veronica* for which we studied multiple populations (*V. serpyllifolia*, *V. hederifolia*, *V. cymbalaria*, *V. persica*; Table 2). One explanation for this large difference of P-O is illustrated by *V. serpyllifolia*. Indeed, a two-fold

difference was observed between the P-O of the German and American populations on the one hand and the Georgian population on the other (Table 2). The most likely reason for this large variation in P-O (60%) within *V. serpyllifolia* is the presence of two varieties (Martínez-Ortega & Rico, 2001): *V. serpyllifolia* var. *humifusa* (few, large, blue flowers; high altitude; large P-O) and var. *serpyllifolia* (many, small, white flowers; low altitude; small P-O). Similar situations may be found in *Veronica orientalis*, in which the variation in ovule number is in line with genetic and taxonomic differences (Albach *et al.*, in prep.; Table 2) and in *V. gentianoides*, in which several taxonomic entities are present (Tumadzhanov *et al.*, 1972; Albach unpubl.; Table 2). Subspecies or varieties within species appear to be an important indication of the potential for large intraspecific variation in P-O and precise taxonomic determinations should accompany detailed investigations in P-O variability.

Ploidy

The second factor influencing P-O variation is intraspecific variation in ploidy. However, the impact of ploidy on P-O is difficult to disentangle from taxonomic differences and supplementary analyses are necessary to define a clear trend (Fukuhara, 2000; Tate & Simpson, 2004). *Veronica cymbalaria* has been shown to include tetraploid and hexaploid individuals indistinguishable morphologically (Fischer, 1975) with hexaploids producing more pollen and having a higher P-O than tetraploids (Table 2). However, recent phylogeographic analyses demonstrated that our tetraploid and hexaploid samples belong to different phylogroups, the tetraploid to the southern Turkish and the hexaploid to the western Turkish group sensu Albach (2007). Similar cases may be found in *V. orientalis* (Albach *et al.*, *in prep.*) and *V. gentianoides* (Tumdzhanov *et al.*, 1972; Albach *et al.*, 2008).

Environment

Biotic factors are not the only factors that can strongly influence intraspecific variation in P-O, some abiotic factors may also act directly on pollen and ovule production (Cruden, 2000). Intraspecific variation of P-O as a consequence of a particular environmental condition has previously been studied in field (Vasek & Weng, 1988; Delesalle *et al.*, 2008) and greenhouse experiments (Vasek & Weng, 1988; Gallardo *et al.*, 1994; Mazer *et al.*, 2009). Similarly, our greenhouse experiment on *V. persica* showed that higher levels of light significantly increased pollen and ovule production and marginally non-significantly decreased P-O ($p = 0.059$; Table 3_A). However, a greater sample size or a greater variation in light intensity could probably give different conclusions. Previous experiments on *Cucurbita* demonstrated that

soil phosphorus levels also played a role in the number of ovules and therefore probably also affects P-O (Lau & Stephenson, 1994). If a single environmental parameter, e.g. light or soil phosphorus, is able to directly modify pollen or ovule production and thus the P-O of a species, completely different habitats with different environmental parameters (humidity, temperature, altitude, soil) would be certain to affect P-O in widely distributed species, as observed in our paired-population investigation. Significant differences of P-O were found between accessions located in different habitats (botanical garden / field) regardless of their phylogeny and indicate that P-Os obtained from field specimens are higher than those from garden (Table 3_B) or greenhouse plants as already noted by Mazer *et al.* (Mazer *et al*., 2009 compared to Vasek & Weng, 1988). The botanical garden specimens could have a lower P-O due to the potential negative impact of inbreeding caused by lower population diversity, as observed in *Mimulus* (Phrymaceae; Ritland & Ritland, 1989) or *Veronica filiformis* (Scalone & Albach, *in prep.*) while the lower P-Os obtained during greenhouse experiments should mainly be due to the effect of environmental conditions, e.g. lower levels of light. Unfortunately, there is no way to discriminate here between the genetic impact of inbreeding and the environmental impact of the non-natural habitat in garden without further experiments.

Finally, intraspecific variation in P-O induced by taxonomic, ploidy level or environmental factors could play an important role in the evolution of sexual reproductive systems and should be investigated in more detail in *Veronica*. We, therefore, see a high interest to investigate intraspecific variation in P-O in more details by manipulative experiments as well as in the field.

2.4.2 Variation of P-O across species in Veronica

Based on our sampling of 11 % of the species in the genus, P-O varies 300-fold from the cleistogamous *V. peregrina* (P-O = 7) to the obligate xenogamous *V. lycica* (P-O = 2572; Figure 1 and Table 1). This large variation in P-O represents all five possible categories of the sexual reproductive system established by Cruden (1977) and is much larger than that reported in some entire families (Vasek & Weng, 1988 within Onagraceae; Gallardo *et al.*, 1994 and Lopez *et al.*, 1999 within Fabaceae; Wyatt *et al.*, 2000 within Apocynaceae; Jürgens *et al.*, 2002 within Caryophyllaceae; Wang *et al.*, 2004 within Zingiberaceae; Yang & Guo, 2007 within Orobanchaceae; Chouteau *et al.*, 2006[A] within Araceae).

Diversity

To explain this high diversity of sexual reproductive systems, one needs to consider the presence of a large diversity of life-forms [e.g. small (1cm tall) annuals to 1-1.5m tall herbs, subshrubs or shrubs] adapted to a large variety of habitats and ecological conditions (e.g. alpine regions, aquatic habitats, dry steppes) located around the world although centered on the Northern Hemisphere and although the large section *Hebe* from the Southern Hemisphere is not even considered here. This radiation was accompanied by many changes in the reproductive traits of *Veronica* along the phylogeny (Figure 1), as well as life history (Müller & Albach, 2010) and ploidy level (Albach *et al.*, 2008). Unfortunately, the best available phylogenetic hypothesis for the genus does not allow us to infer the ancestral character of the sexual system due to the heterogeneity present within species belonging to basal subgenera (subgenera *Veronica & Beccabunga*; Figure 1).

Comparisons with previous studies

Previous publications demonstrated that the sexual reproductive system inferred from log $_{P\text{-}O}$ (Cruden, 1977) could sometimes be contradicted by the results of crossing and germination experiments (Ramirez & Seres, 1994; Chouteau *et al.*, 2006[B]). These incongruencies seem to be minor in *Veronica* since i) a strong relationship exists between our conclusions based on P-O and the conclusions from the crossing experiments extracted from the literature ($r = 0.848$) and since ii) differences between the conclusions based on the two separate datasets (Table 4) within primary and secondary reproductive characters were not found. The lack of such problematic cases shows that inferences of the sexual reproductive systems based on P-O are trustworthy. One only needs to be aware that species reported to be self-compatible can be found to be facultatively xenogamous based on P-O measurements (e.g., *V. cusickii*, *V. montana*).

Bias

This fact partly explains why *Veronica* seems to contain a larger percentage of outcrossing species than would be assumed from published crossing experiments. Alternatively but not mutually exclusive, the difference in the proportion of selfers and outcrossers is probably induced by biases in the choice of species to cross. Researchers crossed *Veronica* species to which they had easy access (weeds of ruderal habitats, which tend to be selfing) instead of species from inaccessible environments (e.g. species endemic to alpine habitats, which are outcrossing) thus leading to an overestimation of selfing species. Finally, although sampling

location and methodology differ between our study and that of Cruden (1977), P-Os of the two common species (*V. arvensis*; *V. serpyllifolia*) are identical (italics in Table 2). This congruence has also been observed in studies of other groups (Chouteau *et al.,* 2006[B]; Wang *et al.,* 2004) and supports our conclusions on the sexual reproductive systems of *Veronica* and the repeatability of P-O measurements within *Veronica*.

2.4.3 Correlations

The study of P-O and its correlates is complex due to inter-connections between and within primary and secondary reproductive traits. Numerous authors have investigated correlations within families and tried to find universal rules (Cruden & Lyon, 1985; Ritland & Ritland, 1989; Vonhoff & Harder, 1995; Parachnowitsch & Elle, 2004; Chouteau *et al.,* 2006; Götzenberger *et al.,* 2006, 2007; Cruden, 2009). In the following, these hypotheses will be discussed in the light of the results found in *Veronica*.

Trade-offs in Veronica

The sex allocation theory affirming the existence of limitations in sexual investments within plants is supported by the detection of size-number trade-offs in gametophytes (Vonhof & Harder, 1995; Lopez *et al.,* 1999; Aguilar *et al.,* 2002; Parachnowitsch & Elle, 2004; Chouteau *et al.,* 2008). Similar to other families or genera, *Veronica* exhibits a strong pollen size - number trade-off and a slight seed size - ovule number trade-off confirming, at first sight, the assumptions of the sex allocation theory on the limits of the sexual resources. However, the absence of a significant negative correlation between pollen size and P-O partially contradicts this conclusion and seems to suggest that a new consensual theory should be found as suggested by Götzenberger (2008). This absence of a correlation between pollen size and P-O is in conflict with almost all previous reports based on family-wide surveys (Vonhof & Harder, 1995; Lopez *et al.,* 1999; Aguilar *et al.,* 2002; Chouteau *et al.,* 2006[A]), but is congruent with similar conclusions obtained in the closely related genus, *Plantago* (Hammer, 1978). The association of relationships between P-O and pollen number and between pollen number and pollen size should theoretically lead to a negative relationship between P-O and pollen size. Originally, Cruden *et al.* (1985[B]) predicted that this relationship exists and that pollen size is correlated with P-O and also with style length, since larger pollen grains have the resources to grow through longer styles. However, the absence of a negative relationship between pollen size and P-O (Charnov, 1982; Cruden & Lyon, 1985[A]; Figure 2) and the lack of a positive relationship between pollen size and style length contradicts the

predictions of Cruden and his pollination efficiency hypothesis in *Veronica* (Cruden & Miller-Ward, 1981; Cruden & Lyon, 1985; Cruden, 2009). Pollen size in *Veronica*, therefore, seems to be independent of nutritional needs of the pollen tube growing through styles. With regards to P-O, the absence of a relationship between P-O and pollen size could be related to the evolution of ovule number in *Veronica* (see below), as suggested by Gallardo *et al.* (1994) in *Astragalus*. These authors suggested that ovule number is very conservative and the ovules are too costly to be affected by inconstancies of breeding system evolution (Gallardo *et al.*, 1994). The strong, positive correlations in secondary reproductive characters (corolla size, stamen and style lengths; Figure 2) demonstrate that all parts of the *Veronica* flower (corolla, androecium, gynoecium) increase allometrically with P-O ($r_{corolla\ /\ P\text{-}O} = 0.429$; $p = 0.0016$; Figure 2). The correlations of P-O with each reproductive traits are complex because of missing data and need to be clarified by investigating further characters such as stigma depth (Cruden, 2009) and by the investigation of the impact of the phylogeny on the relationships between reproductive traits (Götzenberger, 2008).

Sex allocation in Veronica

The comparisons between selfing and outcrossing *Veronica* species for six floral traits confirm that outcrossing species of *Veronica* produce more pollen grains, have a larger corolla, longer styles and stamens and a higher P-O than selfing species in accordance with previous studies in other taxa (Cruden, 1976, 1977; Gallardo *et al.*, 1994; Wang *et al.*, 2004; Table 4). These differences in resource allocation for primary reproductive traits between selfers and outcrossers, regardless of life history (Table 4 & 5_A) are in contrast to conclusions made for secondary reproductive traits (Table 4 & 5_A). The presence of different life histories in *Veronica* is a crucial aspect of our study and started our interest in these investigations. Studies comparing annuals and perennials species within a genus are rare. The perennial *Veronica* species invested more resources in their sexual organs and gametes (pollen and ovule numbers, style and stamen lengths) than the annuals although annuals produced bigger seeds (Table 5_A) as also detected in the related genus *Plantago* (Primack, 1979). Moreover, the production of female gametophytes (ovules) seems not to be affected by the sexual reproductive system within *Veronica* and particularly within the annual species since i) no significant correlation between the two traits was found (ovule number - sexual systems: $r = 0.098$, $p = 0.2910$) and ii) no significant difference was observed in annuals for ovule production. This absence of a relationship between sexual system and ovule number has already been noticed in other studies (Preston, 1986; Vasek & Weng, 1988; Affre &

Thompson, 1998). Instead, ovule number appears to be less evolutionary labile than the reproductive system, since i) the correlation between the evolution of ovule number and phylogenetic history is extremely high (λ =0.902; Figure 2) and ii) more than 90% of the variance in ovule number is related to the species level (Table 3_B), as also found by Jürgens *et al.* (2002) in Caryophyllaceae. The negative correlation between ovule number and corolla size (r = - 0.254, p = 0.0503) suggests a resource trade-off, as already noted by Parachnowitsch & Elle (2004) in *Collinsia parviflora* (Plantaginaceae), but contrasts with the results of studies of other families (Stanton & Preston, 1988; Mione & Anderson, 1992). The increasing allocation to corolla biomass and the constraints imposed by allometric growth seem to decrease ovule formation in *Veronica* and possibly more generally in Plantaginaceae.

To conclude, this study showed the large variation of sexual reproductive system within *Veronica* and provided the basis for further studies in this genus. Inference of the type of sexual reproductive system from P-O corresponded closely to inferences based on much more time-consuming tests of self-compatibility by crossing. P-O appears to be a powerful, cheap, repeatable and fast tool in investigations of plant reproduction. However, more studies under controlled environmental conditions as well as analyses of intraspecific variation are necessary to improve our understanding of the evolution of the sexual system and to determine the causes of variation in P-O. The importance of intraspecific variation for the evolution of the reproductive system and the diversification of the corresponding taxa seem to be particularly worthy of further investigation. Our study offers some initial hypothese that await confirmation from detailed studies on intraspecific relationships. More information on the causes of intraspecific variation will certainly prove important to the understanding of macroevolutionary patterns in the evolution of the sexual reproductive system.

2.5 ACKNOWLEDGEMENTS

We thank Prof. J.W. Kadereit for his guidance during this project. We are grateful to Dr. S. Shetekauri, Mr. T. Shetekauri and Mr. V. Kistauri for their assistance during the collection trip in Georgia. This study was supported by the Deutsche Forschungsgemeinschaft [AL632 / L-1] and the Förderung der wissenschaftlichen Forschung [P18598].

2.6 REFERENCES

Affre L. and Thompson J.D. (1998) Floral traits in four *Cyclamen* (Primulaceae) species. *Plant Systematics and Evolution* **212:** 279-293.

Aguilar R., Bernardello G., Galetto L. (2002) Pollen-pistil relationship and pollen size-number trade-off in species of the tribe *Lycieae* (Solanaceae). *Journal of Plant Research* **115:** 335-340.

Albach D.C. (2007) The use of AFLP and sequence data in the phylogenetic analysis of polyploids: multiple origins of *Veronica cymbalaria* (Plantaginaceae). *New Phytologist* **176:** 481-198.

Albach D.C. and Chase M.W. (2001) Paraphyly of *Veronica* based on sequences from the internal transcribed spacers (ITS) of nuclear ribosomal DNA. *Journal of Plant Research* **114:** 9–18.

Albach D.C. and Chase M.W. (2004) Incongruence in Veroniceae (Plantaginaceae): Evidence from two plastid and a nuclear ribosomal DNA region. *Molecular Phylogenetics and Evolution* **32:** 183–197.

Albach D.C., Martinez-Ortega M.M., Chase M.W. (2004[A]) *Veronica*: parallel morphological evolution and phylogeography in the Mediterranean. *Plant Systematics and Evolution* **246:** 177–194.

Albach D.C., Martinez-Ortega M.M., Fischer M.A., Chase M.W. (2004[B]) A new classification of the *Veroniceae* – Problems and possible solution. *Taxon* **53:** 429-452.

Albach D.C., Meudt H.M., Oxelman B. (2005) Piecing together the "new" Plantaginaceae. *American Journal of Botany* **92:** 297-315.

Albach D.C. and Meudt H.M. (2010) Phylogeny of *Veronica* in the Southern and Northern Hemispheres based on plastid, nuclear ribosomal and nuclear low-copy DNA. *Molecular Phylogenetics and Evolution* **54:** 457-471.

Beatus R. (1935) Genetische und zytologische Untersuchungen in der *Veronica*-Gruppe *Fruticulosae* sekt. *Veronicastrum*. *Flora* **130:** 153-175.

Charlesworth D. and Charlesworth B. (1981) Allocations of resources to male and female functions in hermaphrodites. *Biological Journal of the Linnean Society* **15:** 57-74.

Charnov E.L. (1982) The theory of sex allocation. Princeton, NJ, USA.

Chouteau M., Barabé D., Gibernau M. (2006[A]) A comparative study of inflorescence characters and pollen-ovule ratios among the genera *Philodendron* and *Anthurium* (Araceae). *International Journal of Plant Sciences* **167:** 817-829.

Chouteau M., Barabé D., Gibernau M. (2006[B]) Pollen-ovule ratios in some Neotropical Araceae and their putative significance. *Plant Systematics and Evolution* **257:** 147-157.

Chouteau M., Gibernau M., Barabé D. (2008) Relationships between floral characters, pollination mechanisms, life forms, and habitats in Araceae. *Botanical Journal of the Linnean Society* **156:** 29-42.

Correns C. (1920) Vererbungsversuche mit buntblättrigen Sippen. III. *Veronica gentianoides albocincta*. *Sitzungsberichte der Preussischen Akademie der Wissenschaften zu Berlin* 212-217.

Cruden R.W. (1976) Intraspecific variation in pollen-ovule ratios and nectar secretion: Preliminary evidence of ecotypic adaptation. *Annals of the Missouri Botanical Garden* **63:** 277-289.

Cruden R.W. (1977) Pollen-ovule ratios: a conservative indicator of breeding systems in flowering plants. *Evolution* **31:** 32-46.

Cruden R.W. (2000) Pollen grains: why so many? *Plant Systematics and Evolution* **222:** 143-165.

Cruden R.W. (2009) Pollen grain size, stigma depth, and style length: the relationships revisited. *Plant Systematics and Evolution* **278:** 223-238.

Cruden R.W. and Kinsman S. (1976) Pollination, fecundity, and the distribution of moth-flowered plants. *Biotropica* **8:** 204-210.

Cruden R.W. and Lyon D.L. (1985^{A}) Patterns of biomass allocation to male and female functions in plants with different mating systems. *Oecologia* **66:** 299-306.

Cruden R.W. and Lyon D.L. (1985^{B}) Correlations among stigma depth, style length, and pollen grain size: do they reflect function or phylogeny. *Botanical Gazette* **146:** 143-149.

Cruden R.W. and Miller-Ward S. (1981) Pollen-ovule ratio, pollen size, and the ratio of stigmatic area to the pollen-bearing area of the pollinator: an hypothesis. *Evolution* **35:** 964-974.

Delesalle V.A., Mazer S.J., Paz H. (2008) Temporal variation in the pollen : ovule ratios of *Clarkia* (Onagraceae) taxa with contrasting mating systems: field populations. *Journal of Evolutionary Biology* **21:** 310-323.

Fukuhara T. (2000) Variation of pollen and ovule parameters among different ploidy levels of *Corydalis* (Fumariaceae). *Plant Systematics and Evolution* **224:** 1-12.

Gallardo R., Dominguez E., Munoz J.M. (1994) Pollen-ovule ratio, pollen size, and breeding system in *Astragalus* (Fabaceae) subgenus *Epiglottis*: a pollen and seed allocation approach. *American Journal of Botany* **81:** 1611-1619.

Götzenberger L. (2008) Comparative biology of the pollen-ovule ratio. Halle-Wittenberg, Martin-Luther-University

Götzenberger L., Durka W., Kühn I., Klotz S. (2006) The relationship between the pollen-ovule ratio and seed size: a comparative test of sex allocation hypothesis. *Evolutionary Ecology Research* **8:** 1101-1116.

Götzenberger L., Durka W., Kühn I., Klotz S. (2007) The relationship between the pollen-ovule ratio and pollen size: another comparative test of a sex allocation hypothesis. *Evolutionary Ecology Research* **9:** 1145-1161.

Hammer K. (1978) Entwicklungstendenzen blutenokologisches Merkmale bei *Plantago*. *Flora* **167:** 41-56.

Härle A. (1932) Die Arten und Formen der *Veronica*-Sektion *Pseudolysimachia* Koch auf Grund systematischer und experimenteller Untersuchungen. *Bibliotheca Botanica* **26:** 1-86.

Hong D.-Y. (1984) Taxonomy and evolution of the Veroniceae (Scrophulariaceae) with special reference to palynology. *Opera Botanica* **75:** 5-60.

Jürgens A., Witt T., Gottsberger G. (2002) Pollen grain numbers, ovule numbers and pollen-ovule ratios in Caryophylloideae: correlation with breeding system, pollination, life form, style number, and sexual system. *Sex Plant Reproduction* **14:** 279-289.

Knuth P. (1909) Handbook of flower pollination. Oxford. Clarendon Press.

Lau T.-C. and Stephenson A.G. (1994) Effects of soil phosphorus on pollen production, pollen size, pollen phosphorus content, and the ability to sire seeds in *Cucurbita pepo* (Cucurbitaceae). *Sex Plant Reproduction* **7:** 215-220.

Lehmann E. (1909) Über Zwischenrassen in der *Veronica*-Gruppe A*grestis. Zeitschrift für induktive Abstammungs- und Vererbungslehre* **2:** 145-208.

Lehmann E. (1915) Lichtkeimungsfragen. *Zeitschrift für Botanik* **7:** 560-580.

Lehmann E. (1919) Über die Selbststerilität von *Veronica syriaca. Zeitschrift für induktive Abstammungs- und Vererbungslehre* **21:** 1-47.

Lehmann E. (1922) Über die Selbststerilität von *Veronica syriaca* II. *Zeitschrift für induktive Abstammungs- und Vererbungslehre* **27:** 161-177.

Lehmann E. (1944) *Veronica filiformis* Sm., eine selbsterile Pflanze. *Jahrbücher für wissenschaftliche Botanik* **91:** 395-403.

Lehmann E. and Schmitz-Lohner M. (1954). Entwicklung und Polyploidie in der *Veronica*-Gruppe *Agrestis. Zeitschrift für induktive Abstammungs- und Vererbungslehre* **86:** 1-34.

Lloyd D.G. (1987) Allocations to pollen, seeds and pollination mechanisms in self-fertilizing plants. *Functional Ecology* **1:** 83-89.

Lloyd D.G. and Bawa K.S. (1984) Modification of the gender of seed plants in varying conditions. *Evolutionary Biology* **17:** 255-338.

Lopez J., Rodriguez-Riano T., Ortega-Olivencia A., Devesa J.A., Riuz T. (1999) Pollination mechanisms and pollen-ovule ratios in some *Genisteae* (Fabaceae) from Southwestern Europe. *Plant Systematics and Evolution* **216:** 23-47.

Manukian L. K. (1984) Palinosystematics of the genus *Veronica* L. *Biological Journal of Armenia* **37:** 548-556.

Martinez-Ortega M. M., Sanchez Sanchez J. & Rico E. (2000) Palynological study of *Veronica* Sect. *Veronica* and Sect. *Veronicastrum* (Scrophulariaceae) and its taxonomic significance. *Grana* **39:** 21-31.

Martinez-Ortega M. M. and Rico E. (2001) Seed morphology and its systematic significance in some *Veronica* species (Scrophulariaceae) mainly from the Western Mediterranean. *Plant Systematics and Evolution* **228:** 15-32.

Mazer S.J., Delesalle V.A., Paz H. (2007) Evolution of mating system and the genetic covariance between male and female investment in *Clarkia* (Onagraceae): selfing opposes the evolution of trade-offs. *Evolution* **61:** 83-98

Mazer S.J., Dudley L.S., Delesalle V.A., Paz H., Galusky P. (2009) Stability of pollen-ovule ratios in pollinator-dependent versus autogamous *Clarkia* sister taxa: testing evolutionary predictions. *New Phytologist* **183:** 630-648.

Michalski S.G. and Durka W. (2009) Pollination mode and life form strongly affect the relation between mating system and pollen to ovule ratios. *New Phytologist* **183:** 470-479.

Mione T. and Anderson G. J. (1992) Pollen-ovule ratios and breeding system evolution in *Solanum* section *Basarthrum* (Solanaceae). *American Journal of Botany* **79:** 279-287.

Müller K. and Albach D. (2010) Evolutionary rates in *Veronica* L. (Plantaginaceae): disentangling the influence of life history and breeding system. *Journal of Molecular Evolution* **70:** 44-56.

Pagel M., Meade A., Barker D. (2004) Bayesian estimation of ancestral character states on phylogenies. *Systematic Biology* **53:** 673-684.

Parachnowitsch A.L. and Elle E. (2004) Variation in sex allocation and male-female trade-offs in six populations of *Collinsia parviflora* (Scrophulariaceae s.l.). *American Journal of Botany* **91:** 1200-1207.

Plitmann U. and Levin D.A. (1990) Breeding systems in the Polemoniaceae. *Plant Systematics and Evolution* **170:** 205-214.

Preston R.E. (1986) Pollen-Ovule ratios in the Cruciferae. *American Journal of Botany* **73:** 1732-1740.

Primack R. (1979) Reproductive effort in annual and perennial species of *Plantago* (Plantaginaceae). *The American Naturalist* **114:** 51-62.

Rambaut A. and Drummond A. J. (2007) Tracer version 1.4. available from http://beast.bio.ed.ac.uk/Tracer .

Ramirez N. and Seres A. (1994) Plant reproductive biology of herbaceous monocots in a Venezuelan tropical cloud forest. *Plant Systematics and Evolution* **190:** 129-142.

Ritland C. and Riltand K. (1989) Variation of sex allocation among eight taxa of the *Mimulus guttatus* species complex (Scrophulariaceae). *American Journal of Botany* **76:** 1731-1739.

Ronsted N., Chase M., Albach D., Bello M.A. (2002) Phylogenetic relationships within *Plantago* (Plantaginaceae): evidence from nuclear ribosomal ITS and plastid *trnl-F* sequence data. *Botanical Journal of Linnean Society* **139:** 323-338.

Sánchez Agudo J., Rico E., Sanchez Sanchez J., Martinez-Ortega M. M. (2009) Pollen morphology in the genus *Veronica* L. (Plantaginaceae) and its systematic significance. *Grana* **48:** 239-257.

Scheerer H. (1937) Experimentelle und zytologische Untersuchungen innerhalb der *Veronica*-Gruppe *Pentasepala. Flora* **131:** 287-323.

Schmitz M. (1946) Zur Charakterisierung der Arten der *Veronica*-Gruppe *Agrestis* unter besonderer Berücksichtigung des Polyploidieproblems. Tübingen, University Tübingen.

Stanton M. L. and Preston R. E. (1988) Ecological consequences and phenotypic correlates of petal size variation in wild radish, *Raphanus sativus* (Brassicaceae). *American Journal of Botany* **75:** 528-539.

Tate J.A. and Simpson B.B. (2004) Breeding system evolution in *Tarasa* (Malvaceae) and selection for reduced pollen grain size in the polyploidy species. *American Journal of Botany* **91**: 207-213.

Tumadzhanov I., Beridze R. K., Pogosyan A. (1972) Experience in analysis of *Veronica gentianoides* Vahl s.l. populations along the profile of the Minor Caucasus mountains. *Russkij Botanicheskij Zhurnal* **57**: 1495-1515.

Vasek F.C. & Weng V. (1988) Breeding systems of *Clarkia* sect. *Phaestoma* (Onagraceae). I. Pollen – ovule ratios. *Systematic Botany* **13**: 336-350.

Vieira C.P. and Charlesworth D. (2002) Molecular variation at the self-incompatibility locus in natural populations of the genera *Antirrhinum* and *Misopates*. *Heredity* **88:** 172-181.

Vonhof M.J. and Harder L.D. (1995) Size-Number trade-offs and pollen production by Papilionaceous legumes. *American Journal of Botany* **82:** 230-238.

Wang Y.-Q., Zhang D.-X., Chen Z.-Y. (2004) Pollen histochemistry and pollen : ovule ratios in Zingiberaceae. *Annals of Botany* **94**: 583-591.

Wyatt R., Broyles S.B., Lipow S.R. (2000) Pollen-ovule ratios in Milkweeds (Asclepiadaceae): An exception that probes the rule. *Systematic Botany* **25**: 171-180.

Yang C.-F. and Guo Y.-H. (2007) Pollen-ovule ratio and gamete investment in *Pedicularis* (Orobranchaceae). *Journal of Integrative Plant Biology* **49**: 238-245.

2.7 CONTENTS

- **Figure legends**

Fig 1. Variation of P-O and estimated sexual systems across the *Veronica* phylogeny

The phylogeny is based on previous analyses (Albach *et al.*, 2004; Albach & Meudt, 2010). Sexual reproduction systems, represented by a color code, are estimated by the log $_{P\text{-}O}$ determined in this study and following Cruden´s classification (1977).

Note that data for *Veronica cusickii* were inserted at the phylogenetic position of *V. copelandii*, data for *V. dillenii* at the position of *V. verna*, data for *V. incana* at the place of *V. barrerlieri* and data for *V. vendetta-deae* at the place of *V. baranetzkii*, their respective closest relatives based on literature.

Fig 2. Relationships among sexual reproductive traits (RT) in *Veronica*

Only the significant Pearson product-moment (represented by r =) correlations and the Bayestraits (represented by λ =) correlations above than 0.500 are indicated on the Figure.

Significance level: * = $p \leq 0.05$, ** = $p \leq 0.01$ and *** = $p \leq 0.001$.

- **Table legends**

Table 1. Reproductive traits of *Veronica*

* = species studied in the botanical garden of Mainz, ^{A} = mean values of pollen number and ovule number ± standard deviation with the minimal (min) and the maximal (max) values within parentheses, ^{B} = mean values of P-O with the minimum (min) and the maximum (max) values within parentheses, ^{1} = values in mm, Sex. Sys. = sexual reproductive system based on P-O according to Cruden´s classification (1977), Cle. = cleistogamous species (log $_{P\text{-}O}$ < 1.00), Obl. Aut. = obligate autogamous species (1.00 < log $_{P\text{-}O}$ > 1.80), Fac. Aut. = facultative autogamous species (1.80 < log $_{P\text{-}O}$ > 2.50), Fac. Xen. = facultative xenogamous species (2.50 < log $_{P\text{-}O}$ > 3.30), Obl. Xen. = obligate xenogamous species (log $_{P\text{-}O}$ > 3.30), --- = missing information.

Table 2. Intraspecific variation of pollen number, ovule number and P-O within *Veronica*

n = number of investigated flowers, * = species studied in the botanical garden of Mainz, ^{H} = species studied from herbarium specimens, ^{F} = species studied from field specimens, 4N = tetraploid, 6N = hexaploid, BG = botanical garden, SD = standard deviation; % SD = standard deviation in percentage, Sex. Sys. = sexual reproductive system defined by Cruden classification (Cruden, 1977), --- = missing information, data in italics are data from the literature (Cruden, 1977).

Table 3. Results of the statistical tests

A. for the controlled-environment experiment with different light levels in *V. persica*

T- test (pollen and P-O) and Mann-Whitney-U-test (ovule) comparing the means of each of the observed characters for the two light treatments:

*for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$, ^{NS} for $p > 0.05$.

$N_{flowers}$ = number of flowers; SD = standard deviation; % SD = standard deviation in percentage.

B. for the nested analysis of variance (nested ANOVA) for pollen, ovule and P-O measures obtained from paired populations (botanical garden / field) within six *Veronica* species

Nested ANOVA tests designated by an *F* were performed after square-root transformation (pollen and ovule) and log-transformation (P-O) of the dataset.

Significance level: * = $p \leq 0.05$, ** = $p \leq 0.01$ and *** = $p \leq 0.001$.

df = degree of freedom.

Table 4. Results of the statistical tests of the differences between sexual reproductive systems and the literature data on self-compatibility (C)

One-sample Kolmogorov-Smirnov tests were conducted first to determine the distribution of the dataset (normal / not normal) and Levene-tests were used in order to determine whether the two pairs of categories (selfing / outcrossing and SC / SI) present the same variance (homoscedaticity) or not (heteroscedasticity) within the primary and secondary RT. Thus, T-test (pollen number and size, corolla, style, stamen and seed size and P-O) and Mann-Whitney-U-test (ovule number and Sta-Sty) comparing the means of each observed characters were used depending to the distribution and scedasticity of the data.

Significance level: * = $p \leq 0.05$, ** = $p \leq 0.01$ and *** = $p \leq 0.001$.

RT = reproductive traits, N = total number of investigated species, n = number of species belonging to the category, SC = self-compatible species, SI = self-incompatible species, Pollen $^{\#}$ = pollen number, Pollen ^{W} = pollen width, Pollen^{L} = pollen length,
Data and conclusions in brackets belong to the restricted dataset containing only species known to be SI or SC from crossing data in the literature.

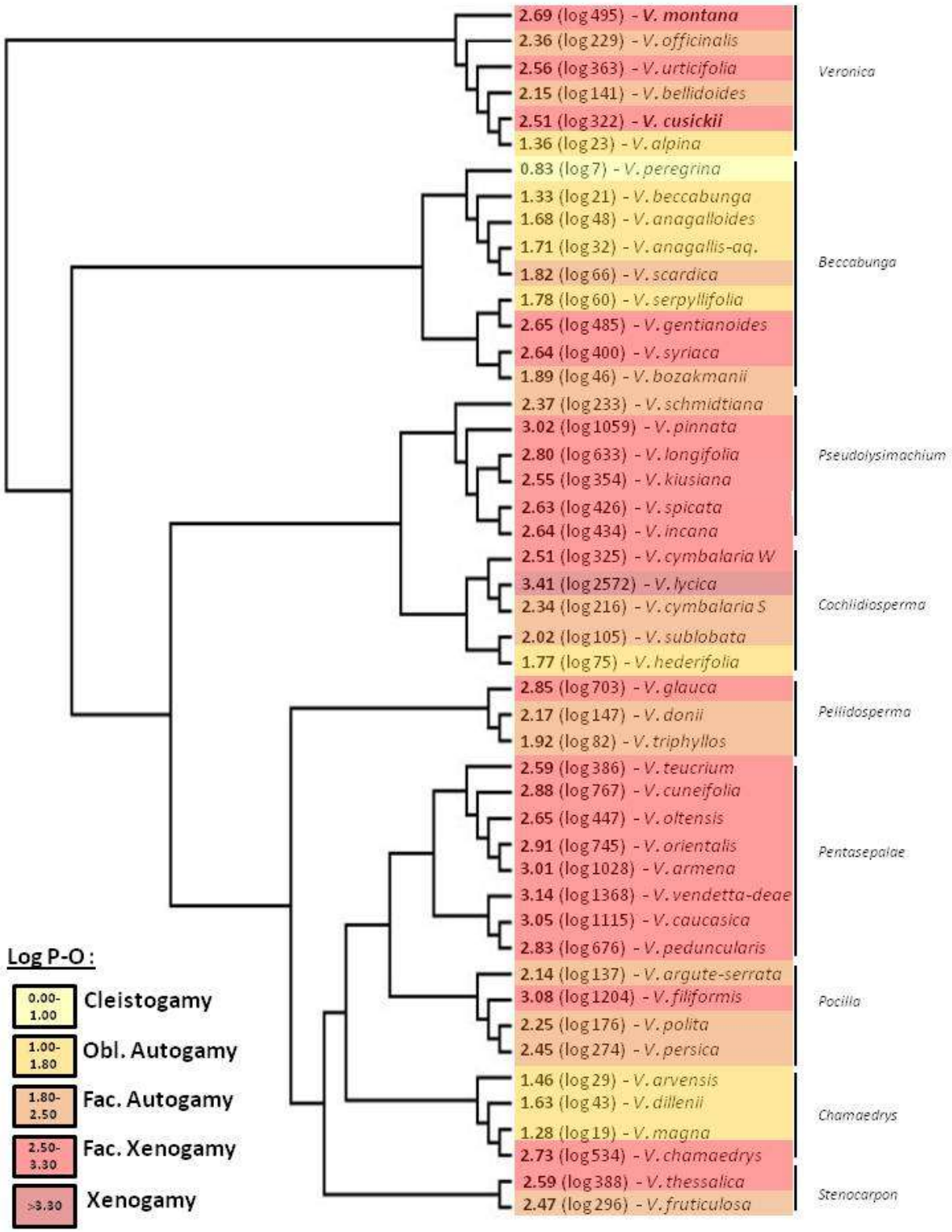
Table 5. Results of the statistical tests for the differences between life histories (annual / perennial) and sexual reproductive systems (selfing / outcrossing)

A. among primary reproductive traits
B. among secondary reproductive traits

T- test (pollen number and size, corolla, style, stamen and seed size and P-O) and Mann-Whitney-U-test (ovule number and Sta-Sty) comparing the means of each observed character for the two life histories or sexual systems
Significance level: * = $p \leq 0.05$, ** = $p \leq 0.01$ and *** = $p \leq 0.001$.
n = number of species; Pollen $^{\#}$ = pollen number; Pollen ^{W} = pollen width; Pollen^{L} = pollen length.

- **Appendix legend**

Appendix 1. Data collected from the literature for the genus *Veronica*
N total = number of known species belonging to the subgenus, % studied = percentage of the known species that were studied in each subgenus, ^{A} = in µm, ^{B} = in mm, --- = unknown data.


2.69 (log 495) - V. montana
2.36 (log 229) - V. officinalis
2.56 (log 363) - V. urticifolia
2.15 (log 141) - V. bellidoides
2.51 (log 322) - V. cusickii
1.36 (log 23) - V. alpina
Veronica
0.83 (log 7) - V. peregrina
1.33 (log 21) - V. beccabunga
1.68 (log 48) - V. anagalloides
1.71 (log 32) - V. anagallis-aq.
1.82 (log 66) - V. scardica
1.78 (log 60) - V. serpyllifolia
2.65 (log 485) - V. gentianoides
2.64 (log 400) - V. syriaca
1.89 (log 46) - V. bozakmanii
Beccabunga
2.37 (log 233) - V. schmidtiana
3.02 (log 1059) - V. pinnata
2.80 (log 633) - V. longifolia
2.55 (log 354) - V. kiusiana
2.63 (log 426) - V. spicata
2.64 (log 434) - V. incana
Pseudolysimachium
2.51 (log 325) - V. cymbalaria W
3.41 (log 2572) - V. lycica
2.34 (log 216) - V. cymbalaria S
2.02 (log 105) - V. sublobata
1.77 (log 75) - V. hederifolia
Cochlidiosperma
2.85 (log 703) - V. glauca
2.17 (log 147) - V. donii
1.92 (log 82) - V. triphyllos
Pellidosperma
2.59 (log 386) - V. teucrium
2.88 (log 767) - V. cuneifolia
2.65 (log 447) - V. oltensis
2.91 (log 745) - V. orientalis
3.01 (log 1028) - V. armena
3.14 (log 1368) - V. vendetta-deae
3.05 (log 1115) - V. caucasica
2.83 (log 676) - V. peduncularis
Pentasepalae
2.14 (log 137) - V. argute-serrata
3.08 (log 1204) - V. filiformis
2.25 (log 176) - V. polita
2.45 (log 274) - V. persica
Pocilla
1.46 (log 29) - V. arvensis
1.63 (log 43) - V. dillenii
1.28 (log 19) - V. magna
2.73 (log 534) - V. chamaedrys
Chamaedrys
2.59 (log 388) - V. thessalica
2.47 (log 296) - V. fruticulosa
Stenocarpon
Log P-O :
0.00-1.00 Cleistogamy
1.00-1.80 Obl. Autogamy
1.80-2.50 Fac. Autogamy
2.50-3.30 Fac. Xenogamy
>3.30 Xenogamy

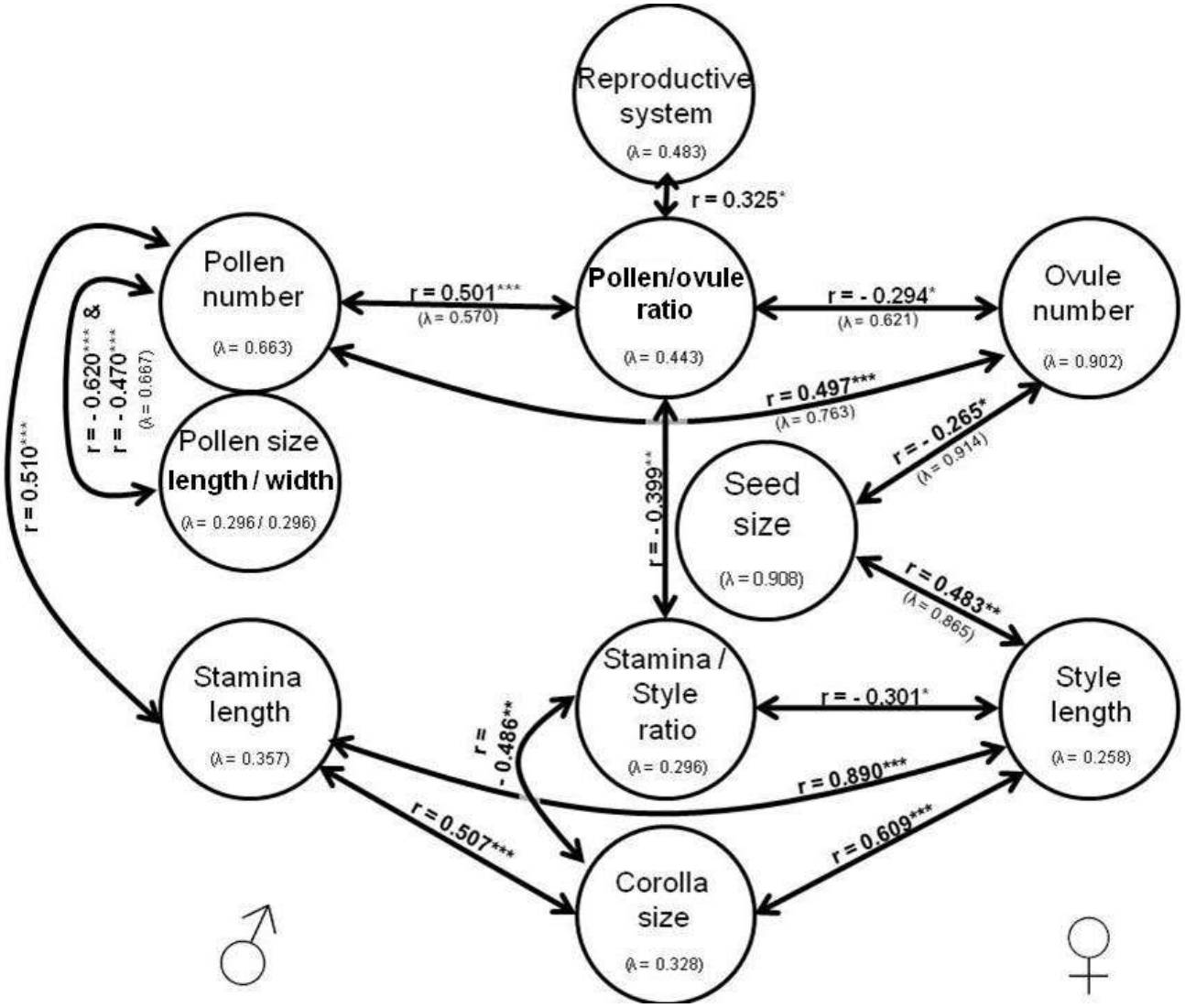

Reproductive system
(λ = 0.483)
r = 0.325*
Pollen number
(λ = 0.663)
r = 0.501***
(λ = 0.570)
Pollen/ovule ratio
(λ = 0.443)
r = - 0.294*
(λ = 0.621)
Ovule number
(λ = 0.902)
r = - 0.620*** &
r = - 0.470***
(λ = 0.667)
r = 0.510***
Pollen size
length / width
(λ = 0.296 / 0.296)
r = 0.497***
(λ = 0.763)
r = - 0.265*
(λ = 0.914)
r = - 0.399**
Seed size
(λ = 0.908)
r = 0.483**
(λ = 0.865)
Stamina length
(λ = 0.357)
Stamina / Style ratio
(λ = 0.296)
r = - 0.301*
Style length
(λ = 0.258)
r = - 0.486**
r = 0.890***
r = 0.507***
r = 0.609***
Corolla size
(λ = 0.328)
♂
♀

Table 1

Species	Pollen [A]	Ovule [A]	P-O [B]	Corolla[1]	Style[1]	Stamen[1]	Sta-Sty	Sex. Sys.	Conclusion
V. montana *	5940 ± 780 (4800 / 6700)	12 ± 0.00 (12 / 12)	495 (400 / 558)	8.0 - 10.0	3.5 - 5.0	3.5 - 5.0	1.00	Fac. Xen.	**outcrossing**
V. officinalis *	4466 ± 712 (3900 / 5500)	20 ± 1.34 (17 / 20)	229 (195 / 324)	6.0 - 8.0	2.5 - 3.0	2.8 - 3.4	1.12 - 1.13	Fac. Aut	**selfing**
V. urticifolia	7620 ± 319 (7200 / 8000)	21 ± 1.09 (20 / 22)	363 (327 / 400)	4.0 - 7.0	4.0 - 7.0	5.0 - 8.0	1.14 - 1.25	Fac. Xen.	**outcrossing**
V. bellidoides	9225 ± 1491 (7300 / 10900)	66 ± 1.91 (64 / 68)	141 (107 / 170)	5.0 - 7.0	1.8 - 2.6	3.2 - 4.0	1.54 - 1.77	Fac. Aut	**selfing**
V. cusickii	9980 ± 926 (9300 / 11600)	31 ± 2.00 (28 / 32)	322 (291 / 414)	10.0 - 13.0	6.0 - 9.0	5.0 - 8.0	0.83 - 0.88	Fac. Xen	**outcrossing**
V. alpina	1200 ± 566 (800 / 2000)	52 ± 0.00 (52 / 52)	23 (15 / 38)	4.0 - 7.0	1.0 - 1.5	2.0 - 2.3	1.50 - 2.00	Obl. Aut	**selfing**
V. peregrina	--- ± --- (--- / ---)	--- ± --- (--- / ---)	7 (--- / ---)	2.0 - 5.0	0.1 - 0.5	1.0	2.00 – 10.00	Cle.	**selfing**
V. beccabunga *	1290 ± 341 (900 / 2000)	61 ± 5.89 (54 / 68)	21 (13 / 37)	5.0 - 6.0	1.5 - 3.0	1.4 - 2.8	0.93	Obl. Aut	**selfing**
V. anagalloides	2840 ± 515 (2188 / 3813)	59 ± 7.15 (52 / 68)	48 (37 / 73)	4.2 - 5.2	0.8 - 1.1	---	---	Obl. Aut	**selfing**
V. anagallis-aquatica	2540 ± 691 (1800 / 3400)	80 ± 2.97 (58 / 66)	32 (27 / 59)	4.0 - 8.0	1.5 - 2.6	3.0 - 3.5	1.40 – 2.00	Obl. Aut.	**selfing**
V. scardica *	2730 ± 999 (1500 / 5100)	42 ± 5.37 (32 / 44)	66 (34 / 159)	4.0 - 5.0	1.2 - 1.6	0.8 - 1.2	0.66 – 0.75	Fac. Aut.	**selfing**
V. serpyllifolia *	3303 ± 1469 (400 / 5600)	55 ± 1.04 (54 / 56)	60 (7 / 104)	5.0 - 8.0	2.0 - 3.0	2.5 - 3.0	1.00 – 1.25	Obl. Aut.	**selfing**
V. gentianoides	16983 ± 4791 (7300 / 25500)	35 ± 4.76 (24 / 40)	485 (183 / 1063)	8.0 - 16.0	4.0 - 8.0	4.0 - 11.0	1.00 – 1.38	Fac. Xen.	**outcrossing**
V. syriaca	12400 ± 738 (11600 / 13300)	31 ± 1.79 (28 / 32)	400 (363 / 475)	10.0 - 12.0	3.5	4.5 - 5.0	1.28 – 1.43	Fac. Xen.	**outcrossing**
V. bozakmanii	525 ± 171 (300 / 600)	12 ± 1.00 (10 / 12)	46 (25 / 60)	4.0 - 7.0	1.2 - 1.6	1.4 - 1.6	1.00 – 1.16	Fac. Aut.	**selfing**
V. schmidtiana *	7454 ± 4181 (1100 / 13300)	32 ± 4.46 (26 / 42)	233 (27 / 439)	13.0 - 14.0	6.0 - 7.0	7.0 - 8.0	1.14 – 1.17	Fac. Aut.	**selfing**
V. pinnata *	21183 ± 3083 (18675 / 24625)	20 ± 0.00 (20 / 20)	1059 (934 / 1231)	---	---	---	---	Fac. Xen.	**outcrossing**
V. longifolia *	55978 ± 6344 (47700 / 67000)	89 ± 4.87 (82 / 98)	633 (487 / 817)	Lv	Lv	Lv	Lv	Fac. Xen.	**outcrossing**
V. kiusiana *	22220 ± 3482 (16700 / 28800)	63 ± 6.06 (52 / 72)	354 (232 / 554)	5.0 - 7.0	7.0	7.0	1.00	Fac. Xen.	**outcrossing**
V. spicata *	30646 ± 3433 (27750 / 34438)	72 ± 2.83 (70 / 74)	434 (299 / 558)	5.0 - 6.0	---	---	---	Fac. Xen.	**outcrossing**
V. incana *	24006 ± 3555 (17938 / 29000)	55 ± 4.16 (52 / 60)	426 (375 / 492)	12.0 - 13.0	3.8 - 4.3	3.1 - 3.8	0.82 – 0.88	Fac. Xen.	**outcrossing**
V. lycica	10288 ± 3255 (6100 / 13700)	4 ± 0.00 (4 / 4)	2572 (1525 / 3425)	12.0 - 16.0	2.8 - 3.5	1.0	0.28 – 0.35	Obl. Xen.	**outcrossing**
V. cymbalaria *	1100 ± 358 (600 / 1400)	4 ± 0.00 (4 / 4)	275 (150 / 350)	6.0 - 12.0	1.0 - 2.0	1.2	0.60 – 1.20	Fac. Aut.	**selfing**
V. sublobata *	420 ± 192 (200 / 700)	4 ± 0.00 (4 / 4)	105 (50 / 175)	4.0 - 5.0	0.3 - 0.6	0.4 - 0.8	1.33	Fac. Aut.	**selfing**
V. hederifolia *	300 ± 122 (200 / 500)	4 ± 0.00 (4 / 4)	75 (50 / 125)	5.0 - 7.0	0.7 - 0.9	0.7 - 1.2	1.00 – 1.33	Obl. Aut.	**selfing**
V. glauca	4080 ± 1995 (1300 / 6900)	6 ± 1.45 (5 / 6)	703 (217 / 1380)	9.0 - 17.0	1.6 - 5.0	1.0	0.20 – 0.62	Fac. Xen.	**outcrossing**
V. donii	2200 ± 1881 (700 / 4700)	15 ± 2.00 (12 / 16)	147 (44 / 392)	20.0 - 24.0	6.0 - 8.0	6.0 - 8.5	1.00 – 1.06	Fac. Aut.	**selfing**
V. triphyllos	1650 ± 354 (1400 / 1900)	20 ± 0.00 (20 / 20)	83 (70 / 95)	5.0 - 7.0	0.7 - 1.5	0.9 - 1.7	1.13 – 1.28	Fac. Aut.	**selfing**
V. teucrium *	8490 ± 3337 (3100 / 13400)	22 ± 2.83 (18 / 24)	386 (129 / 744)	9.0 - 17.0	4.5 - 6.0	4.5 - 6.0	1.00	Fac. Xen.	**outcrossing**
V. cuneifolia	12267 ± 1875 (6400 / 15400)	16 ± 0.00 (16 / 16)	767 (588 / 963)	7.0 - 11.0	3.0 - 5.0	4.0	0.80 – 1.33	Fac. Xen.	**outcrossing**
V. oltensis *	7356 ± 1954 (4950 / 11100)	16 ± 1.67 (14 / 20)	447 (248 / 793)	5.8 - 8.7	1.8 - 3.0	---	---	Fac. Xen.	**outcrossing**
V. orientalis *	8192 ± 2560 (4350 / 13150)	11 ± 4.84 (4 / 16)	745 (272 / 3288)	7.0 - 14.0	3.0 - 5.0	4.0 - 5.0	0.80 – 1.00	Fac. Xen.	**outcrossing**
V. armena *	14389 ± 2659 (10650 / 18200)	14 ± 1.88 (12 / 16)	1028 (666 / 1517)	7.2 - 11.0	2.0 - 3.5	1.7 - 4.7	0.85 – 1.34	Fac. Xen.	**outcrossing**
V. vendetta-deae	16876 ± 3561 (13938 / 25400)	12 ± 0.82 (12 / 14)	1368 (996 / 2117)	7.5 - 11.0	2.5 - 4.5	2.0 - 4.0	0.80 – 0.90	Fac. Xen.	**outcrossing**
V. caucasica *	12106 ± 4578 (5125 / 21125)	11 ± 1.07 (10 / 12)	1115 (427 / 2113)	5.0 - 9.5	1.5 - 3.0	2.0 - 3.3	1.10 – 1.33	Fac. Xen.	**outcrossing**
V. peduncularis	7188 ± 2383 (4125 / 9938)	11 ± 0.74 (10 / 12)	677 (344 / 994)	7.5 - 10.0	2.0 - 3.0	2.3 - 3.5	1.15	Fac. Xen.	**outcrossing**
V. argute-serrata	1375 ± 275 (1100 / 1700)	10 ± 0.00 (10 / 10)	136 (110 / 170)	4.0 - 6.0	1.0 - 1.3	2.5	1.92 – 2.50	Fac. Aut.	**selfing**
V. filiformis *	11563 ± 5153 (3375 / 21300)	10 ± 1.67 (8 / 12)	1205 (281 / 2663)	8.0 - 14.0	3.0 - 4.0	2.1 - 2.5	0.62 – 0.70	Fac. Xen.	**outcrossing**
V. polita *	2902 ± 1340 (1438 / 4687)	16 ± 1.67 (14 / 18)	176 (80 / 335)	4.0 - 7.0	1.0 - 1.6	0.5	0.31 – 0.50	Fac. Aut.	**selfing**
V. persica *	3569 ± 1354 (1000 / 5025)	13 ± 1.35 (12 / 16)	275 (63 / 419)	8.0 - 14.0	2.0 - 2.8	1.8 - 2.4	0.86 – 0.90	Fac. Aut.	**selfing**
V. arvensis *	520 ± 110 (400 / 700)	18 ± 0.89 (18 / 20)	29 (20 / 39)	2.0 - 4.0	0.4 - 0.6	0.3 - 0.5	0.75 – 0.83	Obl. Aut.	**selfing**
V. dillenii	725 ± 206 (500 / 1000)	17 ± 1.00 (16 / 18)	43 (28 / 63)	4.0 - 6.0	0.8 - 1.5	0.8 - 1.5	1.00	Obl. Aut.	**selfing**
V. magna	460 ± 270 (200 / 900)	24 ± 0.00 (24 / 24)	19 (8 / 38)	12.0	4.0 - 4.5	2.5 - 3.0	0.62 – 0.66	Obl. Aut.	**selfing**
V. chamaedrys *	7123 ± 4767 (2300 / 21438)	13 ± 1.55 (10 / 16)	534 (144 / 2144)	10.0 - 14.0	4.0 - 6.0	4.5 - 6.5	1.08 – 1.12	Fac. Xen.	**outcrossing**
V. thessalica	7381 ± 2731 (5400 / 9313)	19 ± 1.41 (18 / 20)	389 (270 / 517)	5.0 - 7.0	3.0 - 5.0	2.5 - 2.9	0.58 – 0.83	Fac. Xen.	**outcrossing**
V. fruticulosa *	9533 ± 1764 (6800 / 12200)	32 ± 0.71 (32 / 34)	296 (200 / 381)	9.0 - 12.0	4.0 - 5.0	3.0 - 4.0	0.75 – 0.80	Fac. Aut.	**Selfing**

Table 2

Species	Locality	Country	n =	Pollen	SD	% SD	n =	Ovule	SD	% SD	P-O	Log_{P-O}	Sex. Sys.	Voucher
***V. officinalis* ***	Mainz, BG	Germany	2	**4500**	713	15.8%	2	**20**	0.000	0.00%	**225.0**	2.35	**F.A.**	Albach 1045, MJG
***V. officinalis* ***	Mainz, BG	Germany	3	**4433**	924	20.8%	3	**19**	1.732	9.12%	**233.3**	2.37	**F.A.**	Albach 1046, MJG
V. anagallis-aquatica [H]	Luberegg	Austria	5	**2540**	691	27.2%	5	**62**	2.966	4.75%	**40.7**	1.61	**O.A.**	Tod & Bauer 12.12.97, WU
V. anagallis-aquatica [F]	Belek (Antalya)	Turkey	9	**5989**	1199	20.0%	9	**97**	6.164	6.33%	**61.7**	1.79	**O.A.**	Albach 2008/ D3-1, MJG
V. serpyllifolia	*Pueblo Co.*	*USA*	---	---	---	---	---	---	---	---	***31.9***	*1.50*	***O.A.***	*Cruden, 1977*
***V. serpyllifolia* ***	Mainz, BG	Germany	8	**2250**	1119	49.7%	6	**55**	1.032	1.87%	**40.7**	1.61	**O.A.**	Albach 1050, MJG
V. serpyllifolia [F]	Bakuriani	Georgia	10	**4355**	956	21.9%	7	**55**	1.069	1.95%	**79.4**	1.90	**F.A.**	Scalone 18, MJG
V. gentianoides [H]	Demirkapi (Bayburt)	Turkey	6	**12683**	3390	26.7%	3	**39**	1.154	2.94%	**325.2**	2.51	**F.X.**	Albach 895, WU
V. gentianoides [H]	Demirkapi (Bayburt)	Turkey	5	**16040**	3578	22.3%	1	**40**	0.000	0.00%	**401.0**	2.60	**F.X.**	Albach 895, WU
V. gentianoides [H]	Karabel (Van)	Turkey	5	**22660**	3110	13.7%	5	**40**	0.000	0.00%	**566.5**	2.75	**F.X.**	Albach 700, WU
V. gentianoides [F]	Kazbegi	Georgia	6	**16550**	3408	20.6%	9	**35**	3.741	10.79%	**477.4**	2.68	**F.X.**	Scalone 8, MJG
V. syriaca [H]	Serik (Antalya)	Turkey	5	**12400**	738	6.0%	5	**31**	1.788	5.73%	**397.4**	2.60	**F.X.**	Albach 250, WU
V. syriaca [F]	Side (Antalya)	Turkey	10	**10150**	2087	20.6%	10	**21**	1.350	6.55%	**483.3**	2.68	**F.X.**	Albach 2008/ D3-5, MJG
V. schmidtiana (white) *****	Mainz, BG	Germany	6	**7933**	4205	53.0%	6	**34**	4.750	13.97%	**233.3**	2.37	**F.A.**	Albach 1043, MJG
V. schmidtiana (blue) *****	Mainz, BG	Germany	8	**6975**	4501	64.5%	8	**30**	3.370	11.23%	**232.5**	2.37	**F.A.**	Albach 1043, MJG
***V. hederifollia* ***	Mainz, BG	Germany	9	**167**	71	42.4%	5	**4**	0.000	0.00%	**41.8**	1.62	**O.A.**	Albach 2008/ D2-3, MJG
V. hederifollia [H]	Wolfsgraben	Austria	5	**300**	122	40.8%	5	**4**	0.000	0.00%	**75.0**	1.87	**F.A.**	Albach 861, WU
V. cymbalaria (south-4N) [H]	Perge (Antalya)	Turkey	3	**900**	436	48.4%	3	**4**	0.000	0.00%	**225.0**	2.35	**F.A.**	Albach 251, WU
V. cymbalaria (south-4N) *****	Mainz, BG	Germany	9	**833**	357	42.8%	9	**4**	0.000	0.00%	**208.3**	2.31	**F.A.**	Albach 2008/ D4-3, MJG
V. cymbalaria (west-6N) [H]	Pergamon (Izmir)	Turkey	3	**1300**	100	7.7%	3	**4**	0.000	0.00%	**325.0**	2.51	**F.X.**	Albach 230, WU
***V. orientalis* ***	Mainz, BG	Germany	4	**5800**	1611	27.8%	4	**16**	0.000	0.00%	**362.5**	2.56	**F.X.**	Scalone F, MJG
V. orientalis [F]	Cakirdaba Pass	Turkey	5	**10958**	1391	12.7%	5	**14**	0.000	0.00%	**782.7**	2.89	**F.X.**	Albach 943, MJG
V. orientalis [F]	Tbilisi	Georgia	10	**7779**	2032	26.1%	8	**6**	1.851	30.86%	**1296.7**	3.11	**F.X.**	Scalone 1, MJG
***V. caucasica* ***	Mainz, BG	Germany	2	**13000**	2546	19.6%	---	---	---	---	---	---	---	Scalone G, MJG
V. caucasica [F]	Mleta	Georgia	9	**11206**	4973	44.4%	7	**11**	1.069	9.85%	**1032.1**	3.01	**F.X.**	Scalone 2, MJG
***V. filiformis* ***	Mainz, BG	Germany	10	**11600**	4587	39.5%	5	**10**	1.673	17.43%	**1208.3**	3.08	**F.X.**	Scalone S65, MJG
V. filiformis [F]	Batumi	Georgia	5	**11140**	3035	27.2%	5	**12**	0.000	0.00%	**928.3**	2.97	**F.X.**	Scalone S118, MJG
V. filiformis [F]	Kazbegi	Georgia	10	**12810**	2788	21.8%	10	**12**	1.265	10.90%	**1104.3**	3.04	**F.X.**	Scalone S42, MJG
***V. polita* ***	Mainz, BG	Germany	4	**1766**	472	26.7%	4	**17**	1.914	11.61%	**107.0**	2.03	**F.A.**	Scalone H, MJG
V. polita [F]	Kazbegi	Georgia	5	**4038**	748	18.5%	5	**16**	1.673	10.20%	**246.2**	2.39	**F.A.**	Scalone 12, MJG
***V. persica* ***	Mainz, BG	Germany	9	**2800**	1362	48.6%	7	**14**	1.154	8.25%	**200.0**	2.30	**F.A.**	Albach 2008 / D2-9, MJG
***V. persica* ***	Light treatment	Germany	50	**6956**	2470	35.5%	50	**21**	3.000	15.90%	**329.0**	2.52	**F.X.**	Greenhouse experiment
***V. persica* ***	Control treatment	Germany	47	**5581**	2262	40.5%	47	**15**	1.000	9.50%	**383.0**	2.58	**F.X.**	Greenhouse experiment
V. persica [F]	Kashuri	Georgia	6	**4338**	680	15.7%	4	**12**	0.000	0.00%	**361.5**	2.56	**F.X.**	Scalone 13, MJG
V. arvensis	*Dickinson Co., KS*	*USA*	---	---	---	---	---	---	---	---	***18.1***	*1.26*	***O.A.***	*Cruden, 1977*
V. arvensis	*Johnson Co., IA*	*USA*	---	---	---	---	---	---	---	---	***25.0***	*1.40*	***O.A.***	*Cruden, 1977*
V. arvensis	*Douglas Co, KS*	*USA*	---	---	---	---	---	---	---	---	***28.3***	*1.45*	***O.A.***	*Cruden, 1977*
V. arvensis *	Mainz, BG	Germany	5	**520**	110	21.1%	5	**18**	1.000	6.06%	**28.9**	1.46	**O.A.**	Albach 1044, MJG
***V. chamaedrys* ***	Mainz, Oberolmer	Germany	2	**3350**	778	23.2%	---	---	---	---	---	---	---	Albach 1026, MJG
***V. chamaedrys* ***	Mainz, BG	Germany	1	**6700**	0	0.0%	---	---	---	---	---	---	---	Albach 1025, MJG
***V. chamaedrys* ***	Mainz, MPI	Germany	5	**6340**	3145	49.6%	10	**14**	1.632	11.66%	**452.9**	2.65	**F.X.**	Albach 1025, MJG
V. chamaedrys [F]	Bakuriani	Georgia	9	**12101**	4070	33.6%	6	**13**	1.032	8.15%	**955.4**	2.98	**F.X.**	Scalone 19, MJG

Table 3A		**Pollen**				**Ovule**				**P-O**			
Treatment	$N_{flowers}$		SD	% SD			SD	% SD			SD	% SD	
Light	50	**6956**	2470	35.5%		**21**	3	15.9%		**329**	121	36.8%	
					******				*******				**NS**
Control	47	**5581**	2262	40.5%		**15**	1	9.5%		**383**	157	41.1%	

Table 3B		Characters (percent of variance)					
nested ANOVA source	df	**Pollen**		**Ovule**		**P-O**	
Species	5	*F* = 5.9*	55,00%	*F* = 28.7***	91,47%	*F* = 8.5*	71,50%
Origins	6	*F* = 7.4***	20,21%	*F* = 30.1***	7,06%	*F* = 14.5***	19,80%
Error	98		24,79%		1,47%		8,70%

Table 4

	N_{total}	n	**Selfing**	[n]	[SC]	n	**Outcrossing**	[n]	[SI]	**Significance**	[Sign.]
Primary RT											
Pollen#	45	22	**2742**	[19]	[2967]	23	**14533**	[7]	[20455]	***	[**]
Pollen^{w}	34	20	**27.59**	[18]	[27.37]	14	**25.00**	[6]	[25.47]	**NS**	[NS]
Pollen^{L}	34	20	**30.56**	[18]	[30.31]	14	**27.79**	[6]	[28.42]	**NS**	[NS]
Ovule	45	22	**30**	[19]	[29]	23	**26**	[7]	[39]	**NS**	[NS]
Log P-O	46	23	**1.87**	[20]	[1.93]	23	**2.80**	[7]	[2.73]	***	[***]
Seed	44	21	**1.37**	[19]	[1.40]	23	**1.40**	[7]	[1.33]	**NS**	[NS]
Secondary RT											
Corolla	44	23	**7.3**	[20]	[6.5]	21	**9.7**	[6]	[10.7]	*	[**]
Style	43	23	**2.2**	[20]	[2.0]	20	**4.2**	[5]	[4.6]	***	[**]
Stamen	41	22	**2.4**	[19]	[2.1]	19	**4.1**	[5]	[5.1]	**	[**]
Sty-Sta	41	22	**1.34**	[19]	[1.36]	19	**1.37**	[5]	[1.06]	**NS**	[NS]

Table 5A	n	**Pollen**[#]		n	**Pollen**[w]		n	**Pollen**[L]		n	**Ovule**		n	**log P-O**		n	**Seed**	
Life history	44	***		34	**NS**		34	**NS**		44	**		45	*		43	*	
Annuals	15	**2993**		13	**28,25**		13	**31.70**		15	**15**		16	**2.09**		14	**1.74**	
Selfing	12	1510	***	12	28.42	**NS**	12	31.54	**NS**	12	8	**NS**	13	1.89	**	11	1.74	**NS**
Outcrossing	3	8922		1	30.10		1	34.70		3	7		3	2.95		3	1.70	
Perennials	29	**11964**		21	**25.40**		21	**28.01**		29	**27**		29	**2.49**		29	**1.24**	
Selfing	9	4386	*	8	26.70	**NS**	8	29.24	**NS**	9	21	*	9	1.84	***	9	1.01	**NS**
Outcrossing	20	15375		13	24.61		13	27.25		20	12		20	2.77		20	1.35	

Table 5B	n	**Corolla**		n	**Style**		n	**Stamen**		n	**Sty-Sta**	
Life history	43	**NS**		42	***		40	***		40	**NS**	
Annuals	16	**8.5**		16	**1.9**		15	**1.8**		15	**1.37**	
Selfing	11	7.1	**NS**	13	1.5	**NS**	12	1.7	**NS**	12	1.50	**NS**
Outcrossing	5	11.6		3	3.3		3	2.2		3	0.70	
Perennials	27	**8.6**		26	**3.9**		25	**4.1**		25	**1.61**	
Selfing	11	7.8	**NS**	9	3.1	**NS**	9	3.4	**NS**	9	1.21	**NS**
Outcrossing	16	9.1		20	4.3		16	4.4		16	1.50	

Appendix 1

Species	Subgenus	Pollen size[C]	Seed size[C]	Life History	SC / SI	Origin	Voucher	% studied
V. montana *	Veronica	**30.5 & 30.3**	**1.95**	**Perennial**	**SC**	Bonn (Germany)	Albach 1049, MJG	
V. officinalis *	Veronica	**30.4 & 27.5**	**1.19**	**Perennial**	**SC**	Table 2	Table 2	
V. urticifolia	Veronica	**24.6 & 22.2**	**1.06**	**Perennial**	**---**	Salzburg (Austria)	Till 10.6.1993, WU	
V. bellidoides	Veronica	**30.9 & 30.5**	**1.32**	**Perennial**	**---**	St. Bernard (Italy)	Albach 193, WU	
V. cusickii	Veronica	**--- & ---**	**1.00**	**Perennial**	**SC**	Chinook Pass (USA)	Albach 290, WU	
V. alpina	Veronica	**26.3 & 25.5**	**0.80**	**Perennial**	**SC**	Norbotten (Finnland)	Schneeweiß et al. 2894, 20.7.99, WU	**14%** (N_{total} = 44)
V. peregrine	Beccabunga	**22.6 & 21.4**	**---**	**Annual**	**SC**	Johnson Co. (USA)	see Cruden, 1977	
V. beccabunga *	Beccabunga	**28.3 & 23.3**	**0.50**	**Perennial**	**SC**	Mainz (Germany)	Albach 1051, MJG	
V. anagalloides	Beccabunga	**30.5 & 24.6**	**0.55**	**Annual**	**SC**	Tsodeniskari (Georgia)	Scalone 27, MJG	
V. anagallis-aquatica	Beccabunga	**30.4 & 24.7**	**0.60**	**Perennial**	**SC**	Table 2	Table 2	
V. scardica *	Beccabunga	**--- & ---**	**0.50**	**Mixed**	**SC**	Bernstein (Austria)	Albach 977, WU	
V. serpyllifolia *	Beccabunga	**27.1 & 26.6**	**0.96**	**Perennial**	**SC**	Table 2	Table 2	
V. gentianoides	Beccabunga	**27.0 & 32.0**	**1.67**	**Perennial**	**SI**	Table 2	Table 2	
V. syriaca	Beccabunga	**--- & ---**	**1.20**	**Annual**	**SI**	Table 2	Table 2	
V. bozakmanii	Beccabunga	**--- & ---**	**1.45**	**Annual**	**---**	Berg Aragac (Armenia)	Gadrusiev 29.4.1971, WU	**27%** (N_{total} = 33)
V. schmidtiana *	Pseudolysimachium	**23.0 & 25.0**	**1.00**	**Perennial**	**---**	Tübingen (Germany)	Albach 1043, MJG	
V. pinnata *	Pseudolysimachium	**18.2 & 21.1**	**1.00**	**Perennial**	**---**	Mainz (Germany)	Scalone A, MJG	
V. longifolia *	Pseudolysimachium	**17.6 & 20.9**	**0.95**	**Perennial**	**SI**	Mainz (Germany)	Albach 1047, MJG	
V. kiusiana *	Pseudolysimachium	**--- & ---**	**1.00**	**Perennial**	**---**	Bonn (Germany)	Albach 1040, MJG	
V. spicata *	Pseudolysimachium	**19.0 & 17.0**	**0.90**	**Perennial**	**SI**	Mainz (Germany)	Scalone C, MJG	
V. incana *	Pseudolysimachium	**13.8 & 16.1**	**0.80**	**Perennial**	**---**	Mainz (Germany)	Scalone B, MJG	**21%** (N_{total} = 29)
V. lycica	Cochlidiosperma	**34.7 & 30.1**	**2.25**	**Annual**	**---**	Gömbe (Turkey)	Albach 266, WU	
V. cymbalaria *	Cochlidiosperma	**37.4 & 35.1**	**2.80**	**Annual**	**SC**	Table 2	Table 2	
V. sublobata *	Cochlidiosperma	**32.1 & 29.4**	**2.45**	**Annual**	**SC**	Mainz (Germany)	Albach 1042, MJG	
V. hederifolia *	Cochlidiosperma	**32.3 & 29.8**	**2.80**	**Annual**	**SC**	Table 2	Table 2	**33%** (N_{total} = 12)
V. glauca	Pellidosperma	**--- & ---**	**1.65**	**Annual**	**---**	Rega Spelagio (Greece)	Albach 404, WU	
V. donii	Pellidosperma	**40.4 & 35.9**	**---**	**Annual**	**---**	Yatagan (Turkey)	Albach 239, WU	
V. triphyllos	Pellidosperma	**30.7 & 29.3**	**1.60**	**Annual**	**SC**	Glaslauterriegels (Austria)	Till 17.3.2007, WU	**43%** (N_{total} = 7)
V. teucrium *	Pentasepalae	**34.0 & 31.3**	**1.75**	**Perennial**	**SI**	Mainz (Germany)	Albach 1052, MJG	
V. cuneifolia *	Pentasepalae	**--- & ---**	**1.50**	**Perennial**	**---**	Avlan Gölü (Turkey)	Albach 2008/ D2-9, MJG	
V. oltensis *	Pentasepalae	**--- & ---**	**1.00**	**Perennial**	**---**	Mainz (Germany)	Scalone E, MJG	
V. orientalis *	Pentasepalae	**27.6 & 22.7**	**2.10**	**Perennial**	**---**	Table 2	Table 2	
V. armena *	Pentasepalae	**32.8 & 30.5**	**1.50**	**Perennial**	**---**	Mainz (Germany)	Scalone D, MJG	
V. vendetta-deae	Pentasepalae	**--- & ---**	**2.00**	**Perennial**	**---**	Kazbegi (Georgia)	Scalone 9, MJG	
V. caucasica *	Pentasepalae	**--- & ---**	**1.25**	**Perennial**	**---**	Table 2	Table 2	
V. peduncularis	Pentasepalae	**29.4 & 31.1**	**1.55**	**Perennial**	**---**	Chuasopeli (Georgia)	Scalone 39, MJG	**12%** (N_{total} = 69)
V. argute-serrata	Pocilla	**29.5 & 33.0**	**2.00**	**Annual**	**SC**	Coruh (Turkey)	Schneeweiß & Jang 7798, WU	
V. filiformis *	Pocilla	**30.3 & 28.3**	**1.50**	**Perennial**	**SI**	Table 2	Table 2	
V. polita *	Pocilla	**25.5 & 22.8**	**1.25**	**Annual**	**SC**	Table 2	Table 2	
V. persica *	Pocilla	**38.2 & 29.7**	**1.85**	**Annual**	**SC**	Table 2	Table 2	**14%** (N_{total} = 28)
V. arvensis *	Chamaedrys	**24.2 & 22.2**	**1.20**	**Annual**	**SC**	Mainz (Germany)	Albach 1044, MJG	
V. dillenii	Chamaedrys	**30.5 & 27.5**	**1.25**	**Annual**	**SC**	Bernhardstal (Austria)	J. Greimler 95/71 14.5.1995, WU	
V. magna	Chamaedrys	**--- & ---**	**1.35**	**Perennial**	**---**	Ayder (Turkey)	Albach 976, WU	
V. chamaedrys *	Chamaedrys	**34.3 & 31.6**	**1.37**	**Perennial**	**SI**	Table 2	Table 2	**31%** (N_{total} = 13)
V. thessalica	Stenocarpon	**--- & ---**	**1.15**	**Perennial**	**---**	Mt. Olympos (Greece)	von Sternburg 270706, WU	
V. fruticulosa *	Stenocarpon	**35.5 & 32.5**	**1.38**	**Perennial**	**SC**	Mainz (Germany)	Albach 1048, MJG	**6%** (N_{total} = 33)

“DIVERSITY OF COROLLA COLOR IN *VERONICA*”


Veronica orientalis
Dr. P. Teege, 2008
Veronica aphylla
Dr. P. Teege, 2008
Veronica gentianoides
Dr. P. Teege, 2008
Veronica caucasica
Dr. P. Teege, 2008
Veronica chamaedrys
Dr. P. Teege, 2008

3 SPECIATION AFFECTED BY SEXUAL REPRODUCTION

"Phylogenetics analysis and differentiation of Veronica *subgenus* Stenocarpon *in the Balkan Peninsula."*

by D. Albach[1], M. von Sternburg[1,2], R. Scalone[1,3] and K. Bardy[3]

from:

[1] Institut für Spezielle Botanik und Botanischer Garten, Johannes Gutenberg-Universität Mainz, Germany

[2] Department of Botany, School of Natural Sciences, University of Dublin, Trinity College Dublin, Ireland

[3] Department of Biogeography, Faculty Center Botany, University of Vienna, Austria

§ author for correspondence: D. Albach, Institut für Biologie und Umweltwissenchaften (IBU), Carl von Ossietzky Universität, Carl von Ossietzky-str. 9-11, 26111 Oldenburg, Germany.

Email: dirk.albach@uni-oldenburg.de,

Phone: (0049) - 441 798 3339,

Fax: (0049) - 441 798 3331,

Keywords: AFLP, Balkan Peninsula, chalcone synthase intron, glacial refugia, ITS, leaf morphology, phylogeography, rpoB-trnC spacer, trnL-trnL-trnF-region.

Running head: Phylogenetics analysis of *Veronica saturejoides*

Received: 18 September 2008

Accepted for publication: 27 January 2009

Botanical Journal of the Linnean Society, 2009, 159, 616-636. With 8 figures

3.1 INTRODUCTION

The Balkan Peninsula is the hotspot of biodiversity in Europe for animals (Dzukic & Kaleciz, 2004; Krystufek & Reed, 2004) and plants. About 6530 plant species occur there with about one third of them endemic (Horvat, Glavac & Ellenberg, 1974; Polunin, 1980). Possible factors possibly responsible for this species richness are the high topographic and climatic diversity of the Peninsula (Krystufek & Reed, 2004). This is especially true for the montane to alpine regions of the Balkan Peninsula, which comprise about 70% of the area (Reed, Krystufek & Eastwood, 2004). Phylogeographic analyses of montane to alpine species have resolved in much detail possible refugia and intraspecific structure in alpine species of the

Alps (Schönswetter *et al*., 2005), but such analyses are scarce for the Balkans (Zhang, Comes & Kadereit, 2001; Frajman & Oxelman, 2007; Stefanovic *et al*., 2008). The use of fossil pollen data (Tzedakis *et al*., 2002; Tzedakis, 2004) and DNA markers (Taberlet *et al*. 1998; Petit *et al*., 2003) have revealed major refugia for forest species in the Balkan Peninsula, but the intraspecific geographic structure for alpine species is unknown. Additional phylogeographical analyses of alpine species, for which fossils are generally lacking, are therefore needed to identify distribution patterns and phylogeographic relationships of regions on the Balkan Peninsula. One complicating factor in phylogeographical analyses is gene exchange of the study group with related taxa. The Pleistocene has been shown to be a time of active evolution in alpine species of the Alps and secondary contact during the interglacials has been implied as a reason for hybrid speciation, in particular polyploid speciation (Stebbins, 1984; Gauthier, Lumaret & Bedecarrats, 1998; Brochman *et al*., 2004). Therefore, these processes also need to be considered in phylogeographical analyses of species from the Balkan Peninsula.

One genus with a complex evolutionary history in the Balkan Peninsula is *Veronica* (Albach 2006), a species-rich genus growing in various kinds of habitats from grasslands in the lowlands to forests and up to the alpine zone. Alpine species in the Balkan Peninsula belong either to *V*. subgenus *Veronica* or to *V*. subgenus *Stenocarpon*, which are only distantly related monophyletic groups in the genus *Veronica* (Albach *et al*., 2004, 2005). The alpine species of *V*. subg. *Veronica* present on the Balkan Peninsula are widespread across Europe and populations on the Balkan Peninsula possibly represent old refugial populations (Albach, Schönswetter & Tribsch, 2006), whereas the alpine species of *V*. subg. *Stenocarpon* on the Balkan Peninsula include three to four species endemic to the region and restricted to only a few localities (Fig. 1). The term "locality" is here used for a given area of alpine habitat surrounded by non-alpine habitat at lower elevation, which can in the larger areas harbour several distinct populations. *Veronica erinoides* is found in the mountains of southern Greece. *Veronica thessalica* is scattered across alpine regions of northern Greece, Macedonia, southern Serbia and northern Albania. Finally, *V. saturejoides* is divided into three subspecies of which the type subspecies is distributed throughout the Dinaric Alps from northern Bosnia to Montenegro at altitudes above 1200 m. *Veronica saturejoides* subsp. *munellensis* was known only from Mount Munella in northern Albania, but here we report a second locality from northern Albania based on intensive investigation of herbarium specimens. All collections of this taxon are more than hundred years old and we currently do not know whether this is due to the inaccessibility of the localities or the extinction of the subspecies.

Finally, *V. saturejoides* subsp. *kellereri*, which is often considered a separate species, *V. kellereri*, is restricted to the Pirin and Rila mountains in western Bulgaria. All three species are prostrate, highly branched herbs with small leaves and a dense terminal or pseudo-terminal inflorescence. They all occur on dry stony habitats on calcareous rocks. These similarities have led to some confusion about the taxon limits and misidentification in the older literature and in herbaria (e.g. Velenovsky, 1902). The current taxonomic concept is based mostly on the studies by Contrandriopoulos & Quézel (1965) and Fischer (1970). These studies provided good diagnostic characters for distinguishing *V. erinoides*, *V. thessalica* and *V. saturejoides* but only weak characters to distinguish the three subspecies of *V. saturejoides*. Therefore, a detailed quantitative analysis of the characters suggested to be diagnostic by Fischer (1970) and a search for other diagnostic characters seemed warranted. Unfortunately, the latter remained unsuccessful (von Sternburg, 2007). Furthermore, molecular characters are needed to give a reliable phylogenetic hypothesis about taxon limits, relationships between them and if possible within the taxa. Previous phylogenetic analyses of DNA sequence data (e.g. Albach, 2006) did not sample all species from the Balkan Peninsula. Nevertheless, Albach (2006) and Albach *et al*. (2005) demonstrated that within *V.* subg. *Stenocarpon* the Balkan endemic species *V. thessalica* and *V. saturejoides* are not closely related to *V. fruticulosa* from the Alps as would be expected from the biogeographical perspective but form an independent lineage together with *V. mampodrensis* from the Iberian Peninsula. Besides the deficiencies in taxon sampling, these studies lacked support for most relationships within subgenus *Stenocarpon*. Additional markers are thus required to resolve internal relationships.

Here, we provide a phylogenetic framework for studies on the Balkan endemic species of *V.* subgenus *Stenocarpon*. The subgenus includes 34 species, with seven occurring in Europe, one in Mexico, two in Turkey, three in the Caucasus and more than half of them in restricted areas of Central Asia. All species of the subgenus are perennial and, as far as they have been investigated, diploid ($2n = 16$; Albach *et al*., 2008) that grow in alpine meadows and montane rocky habitats. Little to nothing is known about their breeding system and dispersal mechanisms, although vegetative reproduction by rerooting of broken-off branches is likely. Including all species of the subgenus is extremely difficult, especially since most of the species with the exception of *V. fruticans*, *V. fruticulosa*, *V. ciliata*, *V. macrostemon* and *V. densiflora* are rare plants with narrow distributions. A comprehensive sampling of the subgenus is therefore beyond the scope of the present study. We focused on the European

species and additionally sampled some additional non-European representatives. For the analysis, we investigated DNA sequence data from both the nuclear (chalcone synthase intron - CHSi, ITS) and plastid (*trnL-trnL-trnF*-region, *rpoB-trnC* spacer) genomes and AFLP fingerprints. The present study is the first to employ sequence data from the nuclear CHSi and the plastid *rpoBtrnC* region in phylogenetic analyses of *Veronica*. The intron of the chalcone synthase, one of the central genes in the flavonoid biosynthetic pathway (Ferrer *et al*., 1999), is the first nuclear low copy region to be employed in phylogenetic analyses of *Veronica*. Nuclear low copy genes are valuable phylogenetic markers, although it may be difficult to find appropriate markers due to the lack of universal primers (Sang, 2002). The data additionally allows preliminary insights into the patterns of genetic diversity in four of the five taxa considered here. Leaf size and shape have been measured for all three subspecies of *V. saturejoides* and *V. thessalica* to test if the taxa can be determined using the characters suggested by Fischer (1970). Additionally, we provide genome size estimates to evaluate the possibility of polyploidy being involved in the evolution of the group. The aim of this study was to test the boundaries of taxa from this subgenus in the Balkan Peninsula, detect possible patterns of hybridization and provide a phylogenetic framework for further investigations in the phylogeographical patterns of the species.

3.2 MATERIALS AND METHODS

3.2.1 Plant material

In the preparation of this study, we checked with our co-workers the occurrence of the taxa in more than 50% of all known localities of the taxa (Fig. 1). Plant material of *V. erinoides* (two out of seven known localities), *V. saturejoides* subsp. *saturejoides* (five out of 14 known localities) and subsp. *kellereri* (two populations from one of two known localities) and *V. thessalica* (three populations from two out of five known localities) was collected in the field and stored in silica gel for DNA analyses. For morphological analyses 3–14 individuals were collected as herbarium specimens. *Veronica saturejoides* subsp. *munellensis* was not found during the field trips, because the localities in northern Albania are fairly inaccessible, and herbarium material was too old for DNA sequencing. Additional species of *V.* subgenus *Stenocarpon* were also included in the analyses. *Veronica prostrata* and *V. arvensis* were chosen as outgroups in the sequence analyses, because they belong to related subgenera (Albach, Martínez-Ortega & Chase, 2004; Albach *et al*., 2005) and amplification of CHSi in several other species of related subgenera failed (von Sternburg, 2007). Origin, voucher information and GenBank accession numbers for all sequences of this study are given in

Table 1. In addition to the herbarium specimens collected for this study, herbarium specimens from various European herbaria (B, BM, C, E, G, HAL, K, SOM, W, WU, Z, ZT) were studied (Table 2). Together they comprise 137 specimens. Latitude and longitude of old herbarium specimens were determined using Fuzzy Gazetteer (Kohlschütter, 2005) and Google Earth.

3.2.2 DNA sequencing

Total genomic DNA was extracted from silica gel dried leaf material using the NucleoSpin Plant Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's specifications. The chalcone synthase intron (CHSi) was amplified using primers CHS1F and CHS2RN (Strand, Leebens-Mack & Milligan, 1997) for all individuals. All other regions were amplified for one individual per population. ITS sequences were amplified using the primers ITS A (Blattner, 1999) and ITS4 (White *et al*., 1990) and include ITS1, 5.8S rDNA and ITS2. The *trnL-trnL-trnF* region was amplified with primers c and f of Taberlet *et al*. (1991) and includes the *trnL* intron, 3´ *trnL* exon and *trnL-trnF* spacer. The *rpoB-trnC* spacer was amplified using primers *rpoB* and *trnC-R* (Shaw *et al*., 2005). PCR reactions included 1 min at 94°C, 35 cycles of 18 sec at 94°C, 30 sec at 55°C and 1 min at 72°C with a final extension time of 8 min at 72°C with the exception of an annealing temperature of 60°C for the *rpoB-trnC* spacer. PCR products were separated on 0.8 % TBE-agarose gels and if more than one fragment was present the one corresponding to the expected size was excised and cleaned using the QIAquick™ PCR purification and gel extraction kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocols. Sequencing reactions (10 µl) were carried out using two µl of the BigDye Terminator Cycle Sequencing mix (Applied Biosystems Inc.), 1 µl of the same primers used for the PCR and 1–5 µl of DNA. Both strands were sequenced on a 3730 DNA Analyzer (Applied Biosystems Inc.). Sequences were assembled and edited using Sequencher™4.7.2 (Gene Codes Corp., Ann Arbor, MI, USA). Assembled sequences were manually aligned prior to analysis. Indels were coded for the parsimony analysis with SeqState v. 1.32 (Müller, 2005) using the modified complex indel coding method (Müller, 2006), which was shown to be the best indel coding method available (Simmons, Müller & Norton, 2007).

3.2.3 Sequence analysis

Datasets were analyzed separately and combined. Sequences from 16 individuals were sequenced for all four regions with maximum parsimony and maximum likelihood using

PAUP v.4.0b10 (Swofford, 2002). Parsimony analyses included heuristic search with random taxon addition (100 replicates) and TBR branch swapping. Parsimony bootstrap support was estimated by analyzing 1000 replicates with the same search conditions but 10 replicates of random taxon addition and a tree limit of 50 per replicate. Maximum likelihood analyses involved models estimated by Modeltest v.3.6 (Posada & Crandall, 1998) based on the AIC and heuristic searches using stepwise taxon addition and TBR branch swapping. Likelihood bootstrap support was estimated by analyzing 700 replicates and the same search conditions as for the maximum likelihood analysis. For all four datasets, analyses with specific constraints to test specific relationships in individual analyses (*V. saturejoides* subsp. *saturejoides* either constrained to be the sister to *V. thessalica* or sister to *V. saturejoides* subsp. *kellereri*) were analyzed identically to the standard analyses. Resulting topologies were compared with the results from the unconstrained analyses using the Templeton test as implemented in PAUP. Additionally, all most parsimonious trees from the single data sets were tested with the data set from all other single datasets.

3.2.4 AFLP generation

The AFLP procedure followed Vos *et al*. (1995) with modifications. To test for reproducibility six replicates were included. Genomic DNA was digested with the two restriction endonucleases *Eco*RI and *Mse*I and ligated to double-stranded *Eco*RI and *Mse*I adaptors in one step at 37°C for 3 hours. The reaction mix for circa 0.5 µg template DNA contained 1.1µl T4 DNA ligase buffer (Promega, Mannheim, Germany), 1.1 µl 0.5 M NaCl, 0,55 µl BSA (1mg/ml, New England Biolabs, Frankfurt, Germany), 1.0 µl 50 µM *Mse*I-adapters (genXpress, Wiener Neudorf, Austria), 0.02µl *Mse*I (50U/µl, New England Biolabs), 1.0 µl 5 µM *Eco*RI-adapters (genXpress), 0.0625µl *Eco*RI (80U/µl, Promega), 0.2 µl T4 DNA ligase (3U/µl, Promega) and 1.53 µl ddH2O. Ligated DNA fragments were diluted 10-fold. Preselective and selective amplifications were performed in a thermocycler (GeneAmp PCR System 9700, Applied Biosystems, Darmstadt, Germany) with PCR protocols following Vos *et al*. (1995). The reaction mix for preselective amplification contained 1.14 µl 10× RedTaq PCR reaction buffer (Sigma-Aldrich, Deisenhofen, Germany), 0.2 µl RedTaq PCR reaction mix (1U/µl, Sigma), 0.22 µl 10 mM dNTPs (Applied Biosystems), 0.58 µl 5 µM preselective primers (genXpress), 5,86µl ddH2O and 2 µl diluted product of the restriction-ligation reaction. The PCR product was diluted 10-fold. The reaction mix for the selective amplification contained 1 µl 10× RedTaq PCR reaction buffer (Sigma), 0.2 µl RedTaq PCR reaction mix (1U/µl, Sigma), 0.22 µl 10 mM dNTPs (Applied Biosystems), 0.54 µl 5µM

*Mse*I-primer (genXpress), 0.54 µl 1µM *Mse*I-primer (Applied Biosystems), 5,5 µl ddH2O and 2 µl diluted product of the preselective amplification. The three primer combinations for the selective PCR were *Eco*RI (6-Fam)-ACA / *Mse*I-CAT; *Eco*RI (VIC)-AAG / *Mse*I-CTG and *Eco*RI (NED)-ACC / *Mse*I-CAA. The selective PCR product was purified using Sephadex G-50 Fine (GE Healthcare Bio-Sciences, Uppsala, Sweden) applied to a Multi Screen-HV plate (Millipore, Molsheim, France) in three steps (200 µl each) and packed at 600g for 1 min the first two times and 5 min at the last step. The same rotation speed was used for the 5 min centrifugation of the samples (5 µl of each selective PCR product). 1.2 µl of the elution product was combined with 9.9 µl HiDi formamide (Applied Biosystems) and 0.1 µl internal size standard GeneScan ROX (Applied Biosystems) and run on an ABI 3130x automated capillary sequencer. Raw AFLP data were collected and aligned with the internal size standard using ABI Prism GeneScan analysis software 3.7 (Applied Biosystems). Peaks (i.e. fragments) were scored manually in Genemarker (SoftGenetics, State College, Pennsylvania) as present (1) or absent (0) in a readable region of bands from 75 to 500 bp in length. Each peak superior to an intensity of signal of 1000 was selected and checked for each sample for the selective amplifications with 6FAM- and VIC-dyes. However, this limit was decreased to 500 for NED-dyes because of the low intensity of this amplification.

3.2.5 AFLP analysis

A neighbor-joining analysis including 1000 bootstrap replicates using Nei–Li distances (Nei & Li, 1979) was conducted in TREECON (Van de Peer & De Wachter, 1994). Additionally, two runs of 1000 bootstrap replicates were conducted using parsimony in PAUP4.0b10 (Swofford, 2002) using ACCTRAN character state transformation in heuristic searches with simple taxon addition and TBR branch swapping. The runs differed in their weighting schemes with one run weighting gains 2:1 over losses and one with the reverse weighting. The strategy was shown to detect patterns of hybridization in the AFLP data set by Albach (2007). We used the model-based clustering based on a Bayesian Markov chain Monte Carlo (MCMC) approach as implemented in the program STRUCTURE 2.2 (Pritchard *et al.* 2000) to identify genetically homogeneous groups. An admixture analysis was run with uncorrelated allele frequencies. The appropriate number of groups (*K*) and the most likely assignment of each individual to a certain group were estimated performing 10 runs for each *K* value ranging from 1 to 9 and 1 million MCMC replicates (100 000 additional replicates as burn-in). The structure runs were performed at the Bioportal of the University of Oslo (www.bioportal.uio.no). The optimal number of clusters was determined according to

Rosenberg *et al.* (2002) as implemented in the R-Skript in Structure 2.1-SUM (Ehrich *et al.*, 2006).

3.2.6 Leaf characters

Leaf characters that have been used to distinguish subspecies of *V. saturejoides* were investigated and measured for 137 herbarium specimens of *V. saturejoides* and *V. thessalica* (Table 2). Measurements were either taken using the „Moticam 1000“ digital camera and the software Motic Images Plus v2.0“ (Motic China group Co., Ltd) or under the binocular. The following characters were measured: leaf length, leaf width and position of widest point relative to the length (Fig. 8). For all characters the position of the leaf along the stem (upper, middle, lower position) and maturity of the stem (sterile, flowering, fruiting) was noted. Leaf indentation was measured but could not be quantified reliably. Leaf indumentum was scored qualitatively. Altogether 1029 leaves from 256 individuals were measured. Boxplots were generated using SPSS v15.0 (SPSS Inc.; Chicago, USA), which was also used to test for correlations among characters and significant differences in characters between taxa.

3.2.7 Genome size measurements

Genome sizes of *Veronica saturejoides* subsp. *saturejoides* (two individuals from Cvrasnica Planina; voucher: B. Surina 05.07.2006, WU), *V. saturejoides* subsp. *kellereri* (Pirin Mts., voucher: M. von Sternburg 2.7.2006, WU), *V. thessalica* (Mt. Olimbos; voucher: M. von Sternburg 27.7.2006, WU), and *V. erinoides* (Mt. Giona; voucher: M. von Sternburg 5.7.2006, WU) were estimated using flow cytometry on CyFlow ML (Partec GmbH, Münster, Germany) equipped with a green laser (Cobolt Samba 100mW) using *Pisum sativum* as internal standard following the protocol of Baranyi & Greilhuber (1996) with propidium iodide staining. For the first, silica gel dried material was used, whereas living material was used for the other three taxa.

3.3 RESULTS

3.3.1 DNA sequence analysis

The chalcone synthase intron was sequenced for eight (out of 33) species of *V.* subgenus *Stenocarpon*. Sequencing was not possible from herbarium derived DNA, thus limiting our sampling within the subgenus. We tried DNA from a number of species outside the subgenus but were only successful twice (*V. arvensis* from *V.* subgenus *Chamaedrys* and *V. prostrata* from *V.* subgenus *Pentasepalae*). The CHSi-region is 575-637bp long in the ingroup and up to

770bp in the outgroup and includes 16 scorable indels (Table 3). When sequences within one population were identical, only one sequence has been used in the analyses. Four different alleles was found in *V. thessalica*. Two individuals out of 13 (15%) from one population in Greece were polymorphic at the position differentiating allele A and B and were, therefore, inferred to be heterozygotes. Sequences from all 28 investigated individuals of *V. saturejoides* subsp. *saturejoides* are identical. In *V. saturejoides* subsp. *kellereri* two widely divergent alleles were found. Eight out of 23 individuals were heterozygotes (35%). The analysis of the CHSi dataset resulted in 65 most parsimonious trees (Fig. 2, Table 3).The most parsimonious trees showed the two subspecies of *V. saturejoides* to be sister taxa (90% parsimony bootstrap support (PBS); 87% likelihood bootstrap support (LBS), with *V. erinoides* as sister to the pair (95 PBS, 64 LBS) and *V. thessalica* in a more distant position. The most likely tree had essentially the same topology as the most parsimonious trees (Fig. 2). The ITS analysis resulted in 405 most parsimonious trees (Fig. 3A, Table 3). The strict consensus tree of the most parsimonious trees is to a large extent unresolved, but *V. saturejoides* subsp. *kellereri* is not monophyletic in any of the individual trees. However, none of the most parsimonious trees shows a sister group relationship of *V. saturejoides* subsp. *saturejoides* to *V. thessalica* as in the plastid DNA analyses (see below). The most likely trees (Fig. 3B) do not differ from the most parsimonious trees in well supported relationships. Combining both nuclear datasets (results not shown) resulted in essential the same topology as that of the CHSi analysis. Support for the monophyly of *V. saturejoides* received 61% PBS, support for the clade including *V. macrostemon*, *V. nummularia*, *V. fruticans* and *V. fruticulosa* increased to 77% PBS.

In contrast, the most parsimonious and most likely trees resulting from the analyses of the *rpoBtrnC* dataset (Fig. 4, Table 3) and from the analyses of the *trnL-trnL-trnF* dataset (Fig. 5, Table 3) identified a clade of *V. thessalica* and *V. saturejoides* subsp. *saturejoides* (*rpoB-trnC*: 100 PBS, 98 LBS; *trnL-trnL-trnF*: 82 PBS, 89 LBS) with *V. saturejoides* subsp. *kellereri* as sister to the pair (57 PBS) and *V. erinoides* as sister to these three (52 PBS) in the *rpoB-trnC* parsimony analysis. None of these relationships was found or contradicted by branches supported in the bootstrap analyses of the *trnL-trnL-trnF* dataset. The difference in the inference of the root between parsimony and likelihood already found with ITS was also found in the *rpoB-trnC* dataset, although here the root found in the parsimony analysis (Fig. 4A) is identical to that in the likelihood analysis of ITS (Fig. 3B) and vice versa. The Templeton-tests revealed significant incongruence between the CHSi, ITS and plastid DNA topologies (Table 4). Asking more specifically whether the position of *V. saturejoides* subsp.

saturejoides was responsible for this result, the tests revealed that the ITS and *trnL-trnL-trnF* datasets were not able to reject either position (sister to *V. saturejoides* subsp. *kellereri* or sister to *V. thessalica*), but CHSi and *rpoB-trnC* clearly rejected the alternative non-parsimonious topology. The analysis of the combined dataset (Fig. 6) provided support for the relationship of two or more species in three cases. *Veronica fruticans* and *V. fruticulosa* are shown to be closely related and undifferentiable using these four DNA regions. Sister to these two species is *V. nummularia* (92 PBS, 77 LBS), as also seen in most separate analyses. Finally, monophyly of the clade consisting of *V. thessalica* and *V. saturejoides* subsp. *saturejoides* was as strongly supported (92 PBS, 85 LBS) as in the separate analyses.

3.3.2 AFLP analysis

Overall, 255 polymorphic bands were scored with a maximum of 85 fragments per individual. *Veronica erinoides*, *V. thessalica*, and both subspecies of *V. saturejoides* each had more than 30 private fragments supporting their distinctness from each other. Both subspecies of *V. saturejoides* combined had 14 private fragments with all other combinations of two of these four taxa having between two and seven private fragments. The neighbor-joining analysis (Fig. 7) further confirmed the four well-supported (100% BS) taxa and also found high support (98% BS) for the monophyly of *V. saturejoides* as in the nuclear analysis and moderate support (79% BS) for the sister group relationship of *V. saturejoides* and *V. erinoides*. The parsimony bootstrap analyses supported the same relationships, albeit with lower percentages. No significant difference was seen between weighting schemes. In the admixture analysis excluding the outgroup, the optimal grouping at k = 4 corresponded to the four taxa revealed by all other molecular analysis. With one exception the admixture analysis grouped all samples with more than 99% probability in the appropriate cluster. The only exception, one sample of *V. saturejoides* subsp. *kellereri* grouped for 2.7% with *V. thessalica* at k=4. At k = 3 *V. thessalica* and *V. erinoides* grouped together.

3.3.3 Morphological analyses

The inspection of 137 herbarium specimens demonstrated differences in several leaf characters between the subspecies of *V. saturejoides* and *V. thessalica*. Leaf indumentum is distinct between the three subspecies and is the most stable character to differentiate the subspecies. Leaves of *V. saturejoides* subsp. *kellereri* are pubescent on the whole adaxial side, whereas those of *V. saturejoides* subsp. *saturejoides* is only pubescent on the leaf margin becoming denser towards the base. Leaves of *V. saturejoides* subsp. *munellensis* are sparsely but evenly pubescent on the adaxial side and the leaf margin. *Veronica thessalica* has

glabrous leaves with some cilia at the base. The abaxial side is glabrous in all subspecies and *V. thessalica*. Leaf indentation is strongest in *V. saturejoides* subsp. *munellensis* (1-2mm) but variable within the subspecies. All leaf size characters are correlated at $p<0.001$ using Pearson correlation. Despite the overlap indicated in Fig. 8, subspecies differ significantly ($p<0.001$) in leaf size characters according to the Mann-Whitney-test except for the length between subsp. *kellereri* and subsp. *munellensis*. Leaves of *V. saturejoides* subsp. *kellereri* are relatively small (5.68mm ±1.29 mm) and elliptic to slightly obovate (widest point at 0.42±0.05 of its length; Fig. 8) in the lower parts of sterile shoots becoming roundish towards the top with the reverse tendency on fertile shoots (von Sternburg, 2007). Leaves of *V. saturejoides* subsp. *saturejoides* are longer (8.3 mm ±2.2 mm) and clearly obovate (widest point at 0.38±0.07 of its length; Fig. 8) with the same tendency within shoots and between sterile and fertile shoots (von Sternburg, 2007). Leaf shape in *V. saturejoides* subsp. *munellensis* is identical to that of *V. saturejoides* subsp. *saturejoides* (widest point at 0.39±0.06 of its length) but smaller (5.53mm ±1.45mm; Fig. 8). The best characters to differentiate the subspecies seems to be the width of the leaves (2.31mm±0.57mm in *V. saturejoides* subsp. *munellensis*, 3.17mm±0.82mm in subsp. *saturejoides* and 3.61mm±0.87mm in subsp. *kellereri*), which leads to leaves wider than long in *V. saturejoides* subsp. *kellereri* in contrast to the other subspecies. A difference in the shape of leaves of *V. saturejoides* subsp. *saturejoides* between populations could not be detected (von Sternburg, 2007). Finally, it should be noted that leaf shape should only be compared in plants from the native habitat, because plants exhibit large phenotypic plasticity under different climatic conditions (von Sternburg & Albach, pers. obs.).

3.3.4 Genome size measurements

The estimations of genome sizes revealed all of the investigated species to have the same ploidy level. The two individuals of *Veronica saturejoides* subsp. *saturejoides* have 1C-values of 0,90 pg (CV: 8,2) and 0,84 pg (CV: 5,8), *V. saturejoides* subsp. *kellereri* 0,88 pg (CV: 3,5), . *thessalica* 0,94 pg (CV: 3,2) and *V. erinoides* 0,75 pg (CV: 2,7).

3.4 DISCUSSION

3.4.1 Molecular characters

The chalcone synthase is a single copy gene in *Antirrhinum majus* L., many Brassicaceae and *Hippophae rhamnoides* (Wienand, Sommer & Schwarz, 1982; Koch, Haubold & Mitchell-Olds, 2000; Bartish, Kadereit & Comes, 2006) but may be found in a multigene family in

other taxa (Durbin, McCaig & Clegg, 2000; Matsumura *et al*., 2005). It also seems to be a single copy gene in *V.* subgenus *Stenocarpon*. Most of our sequences did not show polymorphisms and those that did showed a clear additive pattern of alleles sequenced from other individuals, in which we did not find polymorphisms. Thus, the observed polymorphisms can all be explained by heterozygosity at a single locus. This intron has been proposed as a phylogenetically informative region by Strand *et al*. (1997) but in contrast to the gene (e.g., Koch, Haubold & Mitchell-Olds, 2001) has apparently only been used once in a phylogeographical analysis (Bartish *et al*., 2006). It has about the same number of nucleotides as the ITS region in *Veronica* but has only about half of the parsimony informative characters. Nevertheless, it is a valuable phylogenetic marker as indicated by its high consistency and retention indices (Table 3). Notably the region contains many long indels, especially between different subgenera. *Veronica arvensis* has one insertion of 194 bp relative to all other taxa analysed and *V. prostrata* has an insertion of 303 bp relative to most species of *V.* subgenus *Stenocarpon* (277 bp relative to *V. arvensis*, 353 bp relative to *V. saturejoides*). It will be necessary to have more sequences of CHSi from *Veronica* to detect whether large deletions occurred in the members of *V.* subgenus *Stenocarpon* or whether *V. arvensis* and *V. prostrata* independently evolved large insertions in the chalcone synthase intron. The variation is not surprising given the large range of intron size between less than 100bp and several kilobases reported so far (Wang *et al*., 2000). The CHSi is the first nuclear low copy DNA region employed in phylogenetic analyses of *Veronica*. It has been chosen based on an extensive survey of available primer for such DNA regions (von Sternburg, 2007). The difficulty to amplify the region in taxa from other subgenera of *Veronica* demonstrates that the available primers are not even universal in *Veronica*. Three plastid DNA regions have previously been used in phylogenetic analyses of *Veronica*: *rbcL* (Wagstaff *et al*., 2002), *trnL-trnL-trnF* (e.g. Albach *et al*., 2004) and *rps16* intron (Albach & Chase, 2004). Although especially the latter two provided good support for subgenera, they are not variable enough to resolve relationships among closely related species. The *rpoB-trnC*spacer is among the most informative plastid DNA regions (Shaw *et al*., 2005), although this may not be universally true (Goodson, Santos-Guerra, & Jansen, 2006). Its length here (up to 1223 base pairs) is at the upper range reported (Shaw *et al*., 2005). In the combined analysis it contributed 34 potentially parsimony informative characters compared to 23 contributed by the *trnL-trnL-trnF* dataset (48% more). Furthermore, it seems to be better at resolving relationships based on the higher CI/RI-values (Table 3).

3.4.2 Phylogenetic relationships in V. subgenus Stenocarpon

Reconstructing the phylogeny of *V.* subgenus *Stenocarpon* has been problematic due to the short internal branches relative to the terminal branches (Albach *et al*., 2005; Albach, 2006). This has prevented the delimitation of clades within the subgenus and detection of the correct root of the phylogeny using nuclear ribosomal DNA and the plastid *trnL-trnL-trnF* region (Albach, 2006). The use of other markers both from the nuclear and plastid genome helped detect incongruences between the markers but did not provide a reliable phylogenetic hypothesis for the subgenus or a solution to the rooting problem. Using herbarium specimens for sequencing the chalcone synthase intron for further species of *V.* subgenus *Stenocarpon* has been unsuccessful and restricted the number of species in our analysis. The *rpoB-trnC* spacer has provided a useful test of the *trnL-trnL-trnF*-plastid phylogeny but did not resolve the phylogenetic question better. Using AFLP fingerprints in a phylogenetic analysis is limited by two factors. First, AFLP fingerprints require well preserved DNA, which excludes all but the freshest herbarium specimens from such an analysis. Second, homoplasy of bands becomes a problem with increasing genetic distance and, therefore, AFLPs should usually not be used across more distant taxonomic groups. The long branches leading to the terminal taxa and short branches connecting them (Fig. 7) suggest that increasing taxon sampling in an AFLP analysis is unlikely to resolve the relationships of the subgenus. The most important conclusion regarding the phylogeny of the subgenus is therefore the detection of incongruence between the nuclear (especially CHSi) and plastid DNA datasets. Incongruence between nuclear and plastid DNA datasets can have different reasons including hybridization, introgression, lineage sorting or paralogy. We can exclude the possibility that we have sequenced paralogous loci based on the fact that CHS seems to be single copy in *V.* subgenus *Stenocarpon* (see above) and that CHSi, ITS and AFLPs show congruent results with respect to the monophyly of *V. saturejoides*. This congruence also excludes ancient lineage sorting as a likely explanation for the observed relationships. Another explanation would be a hybrid origin of *V. saturejoides* subsp. *saturejoides* from *V. thessalica* and *V. saturejoides* subsp. *kellereri*. A hybrid origin could either be at the diploid level or involve polyploidy. Chromosome numbers are available for all taxa, but *V. saturejoides* subsp. *saturejoides* and all species in the subgenus counted so far are diploid (Albach *et al*., 2008). The genome size measurements reported here also indicate that polyploidy is not involved in the evolution of the subgenus in the Balkan Peninsula. Although the sequence data is compatible with a diploid hybrid origin of *V. saturejoides* subsp. *saturejoides*, the AFLP results contradict such a conclusion. In a hybridization scenario, we would expect both parents to contribute at least

some private markers to the progeny and the admixture analysis to reveal at least some contribution of one genome to the other. However, *V. thessalica* does not share more private markers with *V. saturejoides* subsp. *saturejoides* than with subsp. *kellereri* and the admixture analysis did not find any support for such a scenario. We therefore prefer introgression in *V. saturejoides* subsp. *saturejoides* as discussed in the next paragraph as an explanation for the incongruence.

3.4.3 Evolution in the Balkan Peninsula

The results are compatible with either of two scenarios. Either *V. saturejoides* subsp. *saturejoides* introgressed into *V. thessalica* or *V. saturejoides* subsp. *saturejoides* was introgressed by the plastid of *V. thessalica*. The scenario of *V. saturejoides* subsp. *saturejoides* plastid DNA introgressing into *V. thessalica* cannot be excluded based on the phylogeny but is unlikely given the monophyly and diversity of plastid DNA sequences in *V. thessalica* and the monomorphy of the *V. saturejoides* subsp. *saturejoides* populations with respect to the plastid DNA sequences. Such a scenario would imply that the introgressed plastid DNA would have diversified in *V. thessalica* but not in *V. saturejoides* since the introgression event or the loss of diversity in the latter. A simpler scenario would involve the reverse pathway for the plastid DNA. The clear genetic differences between the two subspecies of *V. saturejoides* and lack of a significant number of shared private AFLP bands imply an older event with drastic range contraction before the cpDNA introgression event and many generations of backcrossing afterwards compatible with a northward extension of the distribution area. Although we did not find a nuclear DNA marker to substantiate an influence of *V. thessalica* in *V. saturejoides* subsp. *saturejoides*, such an influence could be suggested by morphology and hidden in our analysis by the lack of the southernmost populations of *V. saturejoides* subsp. *saturejoides*, northernmost material of *V. thessalica* and any material of *V. saturejoides* subsp. *munellensis*. After the submission of the manuscript, material from Mt. Durmitor, northern Montenegro became available (Table 1). DNA sequences for three of the four markers were identical to that of more northern populations whereas the fourth, the *rpoB-trnC* spacer, differed by a single substitution. Thus, also this population does not reveal an influence from *V. thessalica*. The morphological analyses indicate an intermediacy of *V. saturejoides* subsp. *munellensis* between the other two subspecies (Fig. 8). Leaf characters of *V. thessalica* have not been studied in as much detail, but the range of leaf shapes overlaps with that of *V. saturejoides* subspp. *saturejoides* and *munellensis* and the leaf indumentum resembles that of *V. saturejoides* subsp. *saturejoides*. An involvement of subsp. *munellensis*

in some kind of genetic exchange between the taxa in the southern Dinaric Mountains is possible, but this hypothesis can currently not be tested.

While crucial material from northern Albania, northern Macedonia and southern Serbia is currently lacking, the analyses here still set the scene for a more intensive study of the southern Dinaric populations of taxa involved here. This involves a scenario in which *V. saturejoides* subsp. *saturejoides* was restricted to one or two smaller population in the northern part of its range during the Pleistocene or migrated there only recently. The separation of the two more western populations (4 – Mt. Troglav, 10 – Mt. Velika Vez) from the three more eastern populations (Fig. 7) suggests at least two Pleistocene populations, which could have occurred west and east of the Dinaric ridge.The genetic homogeneity of different populations of *Veronica saturejoides* subsp. *saturejoides* spanning 300 km from northern to northern Montenegro is remarkable, especially considering the diversity found within populations of *V. thessalica* and *V. saturejoides* subsp. *kellereri*. The pattern of southern richness in haplotypes and northern purity for the group in the western Balkan Peninsula is also found in several species of *Edraianthus* (Stefanovic *et al*., 2008). Lack of morphological diversity (von Sternburg, 2007) parallels that of the genetic diversity and suggests that southern populations were not separated from the northern populations for a long time.

In the absence of a reliable method of dating divergence times in *Veronica* using molecular clocks due to the high substitution rate heterogeneity (Müller & Albach, unpubl.), we assume that *V. saturejoides* subsp. *saturejoides* originated in the south of its range, in the southern Dinaric mountains of southern Serbia, Montenegro and northern Albania, rather than towards the north based on the higher taxonomic diversity of the group in the south and the *rpoB-trnC* sequence from Mt. Durmitor that lacks a synapomorphic substitution present in all other individuals from subsp. *saturejoides*. A Pleistocene restriction to the southern Dinaric mountains has been shown for alpine animals (Sotiropoulos *et al*., 2007) and was inferred as the region of speciation in *Edraianthus* (Stefanovic *et al*., 2008). The distribution of the different taxa in *V.* subgenus *Stenocarpon* is furthermore, despite the ecological differences, surprisingly similar to that of the different subgroups of *Fagus* (beech) detected in the southern Balkan Peninsula (Magri *et al*., 2006), which could indicate that montane forests and alpine plants were restricted to similar regions by colder and drier climate in the Pleistocene. Latitudinal and altitudinal migration from these refugia subsequently presented the opportunity for hybridization and potentially formation of new taxa (Frajman & Oxelman, 2007; Stefanovic *et al*., 2008; results presented here) similar to what has been found further

north in other parts of Europe (Comes & Kadereit, 1998). Therefore, it seems to be worthwhile to study the alpine flora and fauna of the southern Dinaric Mountains in more detail to reveal the complexity of its evolution. To conclude, the results presented here allow clear differentiation of four taxa on the Balkan Peninsula with a fifth (*V. saturejoides* subsp. *munellensis*) currently unavailable for DNA analysis. While the species rank for *V. thessalica* and *V. erinoides* is beyond doubt, the data allows both recognition of *V. saturejoides* subsp. *kellereri* at the subspecific and specific rank. We have given criteria for the distinction between these two ranks in a previous study of other species of *Veronica* (Martínez-Ortega *et al*., 2004). Most of these criteria (genetic cohesion, absence of or insignificant extant gene flow, long branches in phylograms or phenograms, high number of genetic autapomorphies) allow the recognition at the species rank. Furthermore, the non-monophyly of *V. saturejoides* in the plastid DNA-phylogenetic trees (Fig. 4, 5) seems to strongly argue for the species rank. However, we feel that leaf pubescence does not suffice as a clearly diagnosable morphological character (Fig. 8) and the pattern in the plastid DNA sequences can be best explained as interspecific introgression. Therefore, we feel that it is most appropriate to use the subspecific rank for *V. saturejoides* subsp. *kellereri*.

3.5 ACKNOWLEDGEMENTS

The project was financed by the Austrian Science Fund (FWF-Fonds zur Förderung wissenschaftlicher Forschung) project P18598 and a EU-SYNTHESYS-project (AT-TAF-2409), which is gratefully acknowledged. Help by D. Peev and V. Vladimirov was instrumental in the plant collection excursions. Additional plant material was provided by B. Frajman, P. Schönswetter and B. Surina. Support in the lab was provided by M. Kever and others at the Institut für Spezielle Botanik, Mainz. We also thank the curators of the herbaria that loaned specimens on loan or provided access to their collections, especially E. Vitek. M. Martínez Ortega and an anonymous reviewer provided valuable comments to the manuscript. We thank J. Kadereit for constant suggestions for improvement from the start of the study to the final manuscript. Finally, the project greatly benefitted from the intellectual support by M. A. Fischer.

3.6 REFERENCES

Albach D. C. (2006)**.** Evolution of *Veronica* (Plantaginaceae) on the Balkan Peninsula. *Phytologia Balcanica* **12:** 231–244.

Albach D. C. (2007). Amplified fragment length polymorphisms and sequence data in the phylogenetic analysis of polyploids: multiple origins of *Veronica cymbalaria* (Plantaginaceae). *New Phytologist* **176**: 481–498.

Albach D. C. and Chase M. W. (2004). Incongruence in Veroniceae (Plantaginaceae): evidence from two plastid and a nuclear region. *Molecular Phylogenetics and Evolution* **32:** 183–197.

Albach D. C., Martínez-Ortega M. M., Chase M. W. (2004). *Veronica*: parallel morphological evolution and phylogeography in the Mediterranean. *Plant Systematics and Evolution* **246:** 177– 194.

Albach D. C., Jensen S. R., Özgökce F., Grayer R. J. (2005). *Veronica*: chemical characters for the support of phylogenetic relationships based on nuclear ribosomal and plastid DNA sequence data. *Biochemical Systematics and Ecology* **33:** 1087–1106.

Albach D. C., Schönswetter P., Tribsch A. (2006). Comparative phylogeography of the *Veronica alpina* complex in Europe and North America. *Molecular Ecology* **15:** 3269–3286.

Albach D. C., Martínez-Ortega M. M., Delgado-Sánchez L., Weiss-Schneeweiss H., Özgökce F, Fischer M. A. (2008). Chromosome numbers in Veroniceae (Plantaginaceae): review and several new counts. *Annals of the Missouri Botanical Garden* **95:** in press.

Baranyi M. and Greilhuber J. (1996). Flow cytometric and Feulgen densitometric analysis of genome size variation in *Pisum*. *Theoretical and Applied Genetics* **92:** 297–307.

Bartish I. V., Kadereit J. W., Comes H. P. (2006). Late Quaternary history of *Hippophae rhamnoides* L. (Eleagnaceae) inferred from chalcone synthase intron (Chsi) sequences and chloroplast DNA variation. *Molecular Ecology* **15:** 4065–4083.

Blattner F. R. (1999). Direct amplification of the entire ITS region from poorly preserved plant material using recombinant PCR. *BioTechniques* **27:** 1180–1186.

Brochmann C., Brysting A. K., Alsos I. G., Borgen L., Grundt H. H., Scheen A. C., Elven R. (2004). Polyploidy in arctic plants. *Biological Journal of the Linnean Society* **82:** 521–536.

Comes H. P. and Kadereit J. W. (1998). The effect of Quaternary climatic changes on plant distribution and evolution. *Trends in Plant Sciences* **3:** 432–438.

Contandriopoulos J. and Quézel P. (1965). A propos de deux veroniques critiques des montagnes grecques: *Veronica erinoides* Boiss. & et *V. thessalica* Benth. *Candollea* **20:** 43–48.

Durbin M. L., McCaig B., Clegg M. T. (2000). Molecular evolution of the chalcone synthase multigene family in the morning glory genome. *Plant Molecular Biology* **42:** 79–92.

Dzukic G. and Kalezic M. L. (2004). The biodiversity of amphibians and reptiles in the Balkan Peninsula. In: Griffiths HI, Krystufek B, eds. *Balkan biodiversity: pattern and process in the European hotspot*. Dordrecht: Kluwer Academic Publishers, 167–192.

Ehrich D. (2006). aflpdat: a collection of R functions for convenient handling of AFLP data. *Molecular Ecology Notes* **6:** 603–604.

Ferrer J. L., Jez J. M., Bowman M. E., Dixon R. A., Noel J. P. (1999). Structure of chalcone synthase and the molecular basis of plant polyketide biosynthesis. *Nature Structural Biology* **6:** 775–784.

Fischer M. (1970). *Veronica quezelii* und *V. saturejoides* Vis. subsp. *munellensis*, zwei neue Sippen der Sektion *Veronicastrum* aus ostmediterranen Gebirgen. *Plant Systematics and Evolution* **118:** 201–205.

Frajman B. and Oxelman B. (2007). Reticulate phylogenetics and phylogeographical structure of *Heliosperma* (Sileneae, Caryophyllaceae) inferred from chloroplast and nuclear DNA sequences. *Molecular Phylogenetics and Evolution* **43:** 140–155.

Gauthier P., Lumaret R., Bedecarrats A. (1998). Genetic variation and gene flow in alpine diploid and tetraploid populations of *Lotus* (*L. alpinus* (D. C.) Schleicher/*L. corniculatus* L.). I. Insights from morphological and allozyme markers. *Heredity* **80:** 683–693.

Goodson B. E., Santos-Guerra A., Jansen R. K. (2006). Molecular systematics of *Descurainia* (Brassicaceae) in the Canary Islands: biogeographic and taxonomic implications. *Taxon* **55:** 671–682.

Horvat I., Glavac V., Ellenberg H. (1974). *Vegetation Südosteuropas*. Stuttgart: G. Fischer.

Koch M. A., Haubold B., Mitchell-Olds T. (2000). Comparative evolutionary analysis of chalcone synthase and alcohol dehydrogenase loci in *Arabidopsis, Arabis* and related genera (Brassicaceae). *Molecular Biology and Evolution* **17:** 1483–1498.

Koch M. A., Haubold B., Mitchell-Olds T. (2001). Molecular systematics of the Brassicaceae: evidence from coding plastidic *matK* and nuclear chs sequences. *American Journal of Botany* **88:** 534–544.

Kohlschütter C. (2005). Fuzzy Gazetteer - FuzzyG v2.1-dev. accessed 2006 & 2007, http://tomcat-dmaweb1.jrc.it/fuzzyg/query/**.**

Krystufek B. and Reed J. M. (2004). Pattern and process in Balkan biodiversity - an overview. In: Griffiths HI, Krystufek B, eds. *Balkan biodiversity: pattern and process in the European hotspot*. Dordrecht: Kluwer Academic Publishers, 1–8.

Magri D., Vendramin G. G., Comps B., Dupanloup I., Geburek T., Gömöry D., Latalowa M., Litt T., Paule L., Roure J. M., Tantau I., van der Knaap W. O., Petit R. J.,

de Beaulieu J. L. (2006). A new scenario for the Quaternary history of European beech populations: palaeobotanical evidence and genetic consequences. *New Phytologist* **171:** 199–222.

Martínez-Ortega M. M., Delgado L., Albach D. C., Elena-Rosselló J. A., Rico E. (2004). Species boundaries and phylogeographic patterns in cryptic taxa inferred from AFLP markers: *Veronica* subgen. *Pentasepalae* (Scrophulariaceae) in the Western Mediterranean. *Systematic Botany* **29:** 965–986.

Matsamura H., Watanabe S., Harada K., Senda M., Akada S., Kawasaki S., Dubouzet E. G., Minaka N., Takahashi R. (2005). Molecular linkage mapping and phylogeny of the chalcone synthase multigene family in soybean. *Theoretical and Applied Genetics* **110:** 1203–1209.

Müller K. (2005). SeqState: primer design and sequence statistics for phylogenetic DNA datasets. *Applied Bioinformatics* **4:** 65–69.

Müller K. (2006). Incorporating information from length-mutational events into phylogenetic analysis. *Molecular Phylogenetics and Evolution* **38:** 667–676.

Nei M. and Li W. H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences USA* **76**: 5269–5273.

Petit R. J., Aguinagalde I., de Beaulieu J. L., Bittkau C., Brewer S., Cheddadi R., Ennos R., Fineschi S., Grivet D., Lascoux M., Mohanty A., Müller-Starck G., Demesure-Musch B., Palmé A., Martin J. P., Rendell S., Vendramin G. G. (2003). Glacial refugia: hotspots but not melting pots of genetic diversity. *Science* **300:** 1563–1565.

Polunin O. (1980). *Flowers of Greece and the Balkans*. Oxford: Oxford University Press.

Posada D. and Crandall K. A. (1998). MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14:** 817–818.

Pritchard J. K., Stephens M., Donnelly P. (2000). Inference of population structure using multilocus genotype data. *Genetics* 155: 945–959.

Reed J. M., Krystufek B., Eastwood W. J. (2004). The physical geography of the Balkans and nomenclature of place names. In: Griffiths HI, Krystufek B, eds. *Balkan biodiversity:pattern and process in the European hotspot*. Dordrecht: Kluwer Academic Publishers, 9–22.

Rosenberg N. A., Pritchard J. K., Weber J. L., Cann H. M., Kidd K. K., Zhivotovsky L. A., Feldman M. W. (2002). Genetic structure of human populations. *Science* 298: 2381–2385.

Sang T. (2002). Utility of low-copy nuclear gene sequences in plant phylogenetics. *Critical Reviews in Biochemistry and Molecular Biology* **37:** 121–147.

Schönswetter P., Stehlik I., Holderegger R., Tribsch A. (2005). Molecular evidence for glacial refugia of mountain plants in the European Alps. *Molecular Ecology* **14:** 3547–3555.

Shaw J., Lickey E. B., Beck J. T., Farmer S. B., Liu W., Miller J., Siripun K. C., Winder C. T., Schilling E. E., Small R. L. (2005). The tortoise and the hare II: relative utility of 21noncoding chloroplast DNA sequences for phylogenetic analysis. *American Journal of Botany* **92:** 142–166.

Simmons M. P., Müller K., Norton A. P. (2007). The relative performance of indel-coding methods in simulations. *Molecular Phylogenetics and Evolution* **44:** 724–740.

Sotiropoulos K., Eleftherakos K., Dzukic G., Kalezic M. L., Legakis A., Polymeni R. M. (2007). Phylogeny and biogeography of the alpine newt *Mesotriton alpestris* (Salamandridae, Caudata), inferred from mtDNA sequences. *Molecular Phylogenetics and Evolution* **45:** 211–226.

Stebbins G. L. (1984). Polyploidy and the distribution of the arctic-alpine flora: new evidence and a new approach. *Botanica Helvetica* **94:** 1–13.

Stefanović S., Lakušić D., Kuzmina M., Mededović S., Tan K., Stevanović V. (2008). Molecular phylogeny of *Edraianthus* (grassy bells; Campanulaceae) based on non-coding plastid DNA sequences. *Taxon* **57**: 452–475.

Strand A. E., Leebens-Mack J., Milligan B. G. (1997). Nuclear DNA-based markers for plant evolutionary biology. *Molecular Ecology* **6:** 113–118.

Swofford D. L. (2002). *PAUP* Phylogenetic Analysis Using Parsimony (*and other Methods)*. Sunderland: Sinauer Associates.

Taberlet P., Gielly L., Pautou G., Bouvet J. (1991). Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* **17:** 1105–1109.

Taberlet P., Fumagalli L., Wust-Saucy A.G., Cosson J. F. (1998). Comparative phylogeography and postglacial colonization routes in Europe. *Molecular Ecology* **7:** 453–464.

Tzedakis P. C., Lawson I. T., Frogley M. R., Hewitt G. M., Preece R.C. (2002). Buffered tree population changes in a quaternary refugium: evolutionary implications. *Science* **297:** 2044–2047.

Tzedakis P. C. (2004). The Balkans as prime glacial refugial territory of European temperate trees. In: Griffiths HI, Krystufek B, eds. *Balkan biodiversity: pattern and process in the European hotspot*. Dordrecht: Kluwer Academic Publishers, 49–68.

Van de Peer Y. and De Wachter R. (1994). TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Computer Applications in the Biosciences* **10**: 569–570.

Velenovsky J. (1902). Planta novae bulgaricae. *Österreichische Botanische Zeitschrift* **52:** 154–156.

von Sternburg M. (2007). Phylogeographie und Differenzierung von *Veronica saturejoides*. Unpublished Diploma Thesis. Johannes Gutenberg-Universität Mainz, Germany.

Vos P., Hogers R., Bleeker M., Reijans M., van de Lee T., Hornes M., Frijters A., Pot J., Peleman J., Kuiper M. (1995). AFLP: a new concept for DNA fingerprinting. *Nucleic Acids Research* **23:** 4407–4414.

Wagstaff S. J., Bayly M. J., Garnock-Jones P. J., Albach D. C. (2002). Classification, origin, and diversification of the New Zealand hebes (Scrophulariaceae). *Annals of the Missouri Botanical Garden* **89:** 38–63.

Wang J. L., Qu L.J., Chen J., Gu H. Y., Chen Z. L. (2000). Molecular evolution of the exon 2 of CHS genes and the possibility of its application to plant phylogenetic analysis. *Chinese Science Bulletin* **45:** 1735–1742.

White T. J., Bruns T., Lee S., Taylor J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand, DH, Sninsky JJ, White TJ, eds. *PCR protocols: a guide to methods and applications*. San Diego: Academic Press, 315–322.

Wienand U., Sommer H., Schwarz U. (1982). A general method to identify plant structural genes among genomic DNA clones using transposable element induced mutations. *Molecular Genetics and Genomics* **187:** 195–201.

Zhang L., Comes H. P., Kadereit J. W. (2001). Phylogeny and quaternary history of the European montane/alpin endemic *Soldanella* (Primulaceae) based on ITS and AFLP variation. *American Journal of Botany* **88:** 2331–2345.

3.7 CONTENTS

- **Figure legends**

Fig 1. Distribution of *Veronica erinoides*, *V. saturejoides* and *V. thessalica* based on herbarium specimens investigated and literature

Smaller symbols indicate populations reported in the literature but not seen as herbarium specimens in this study. Numbers refer to localities as indicated in Table 2. Asterisks mark

populations sampled in the DNA sequence analysis. Smaller circles indicate populations reported in the literature but not seen from herbarium specimens.

Fig. 2. One random tree of the 65 most-parsimonious trees of the analysis of the chalcone synthase intron (CHSi) data set

Arrows indicate branches not present in all most-parsimonious trees and in the maximum likelihood tree. Numbers on the branches indicate parsimony branch lengths (above branch), parsimony bootstrap percentage (below branch, before slash) and likelihood bootstrap percentage (below branch, after slash). Only bootstrap values above 50% are indicated. Numbers in brackets after the names indicate number of individuals in which the specific allele has been found (homozygous/heterozygous individuals). See Figure 1 for information on the localities.

Fig. 3. Results from the analysis of the internal transcribed spacer (ITS) data set

A) one random tree of the 405 most-parsimonious trees.

Arrows indicate branches not present in all most-parsimonious trees.

B) one of the maximum likelihood trees.

The other tree did not differ in topology but in branch lengths. Numbers on the branches indicate parsimony branch lengths (above branch) and bootstrap percentage (below branch). Only bootstrap values above 50% are indicated. See Figure 1 for information on the localities.

Fig. 4. Results from the analysis of the *rpoB-trnC* data set

A) one random tree of the 12 most-parsimonious trees.

Arrows indicate branches not present in all most-parsimonious trees.

B) maximum likelihood tree.

Numbers on the branches indicate parsimony branch lengths (above branch) and bootstrap percentage (below branch). Only bootstrap values above 50% are indicated. See Figure 1 for information on the localities.

Fig. 5. One random tree of the 10 562 most-parsimonious trees from the *trnL-trnL-trnF* data set

Arrows indicate branches not present in all most-parsimonious trees and in the maximum likelihood tree. Numbers on the branches indicate parsimony branch lengths (above branch), parsimony bootstrap percentage (below branch, before slash) and likelihood bootstrap

percentage (below branch, after slash). Only bootstrap values above 50% are indicated. See Figure 1 for information on the localities.

Fig. 6. One of the most-parsimonious trees from the combined analysis of the chalcone synthase intron (CHSi), internal transcribed spacer (ITS), *rpoB-trnC* and *trnL-trnl-trnF*
Arrows pointing downwards indicate branches not present in all most-parsimonious trees. Arrows pointing upwards indicate branches not present in the maximum likelihood tree. Numbers on the branches indicate parsimony branch lengths (above branch), parsimony bootstrap percentage (below branch, before slash) and likelihood bootstrap percentage (below branch, after slash). Only bootstrap values above 50% are indicated. See Figure 1 for information on the localities.

Fig. 7. Neighbor-joining (NJ) tree based on the AFLP data set
Numbers at terminals refer to the localities in Figure 1. Apostrophe after the number indicates amplified fragment length polymorphism (AFLP) replicates. Numbers on the branches indicate bootstrap support (NJ-values above the branch, parsimony 2 : 1/1 : 2-weighted below the branch).

Fig. 8. Boxplot indicating variation in leaf morphological traits among the three subspecies of *Veronica saturejoides* and *V. thessalica*
The box indicates the interquartile (25–75%) range. The bar within the box indicates the mean value. Whiskers below and above the box indicate the whole range of values.

- **Table legends**

Table 1. Information on vouchers and GenBank accession numbers used in this study
Sequences used in the combined analysis are printed in bold. For locality numbers see Figure 1 and Table 2. CHSi = chalcone synthase intron; ITS = internal transcribed spacer.

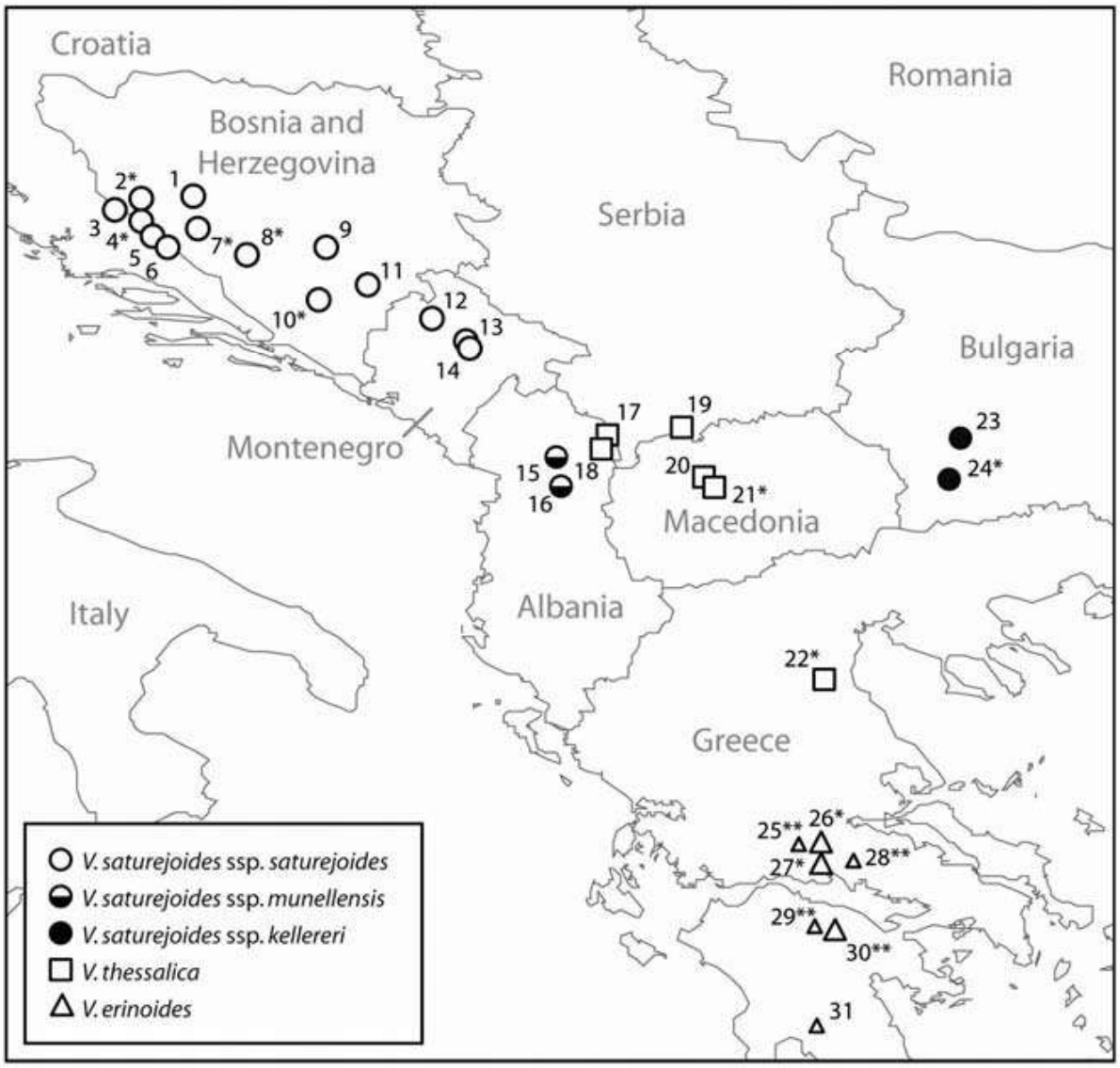
Table 2. Herbarium specimens measured in morphological analyses
Numbers at the beginning of the lines refer to locality numbers used in Figure 1.

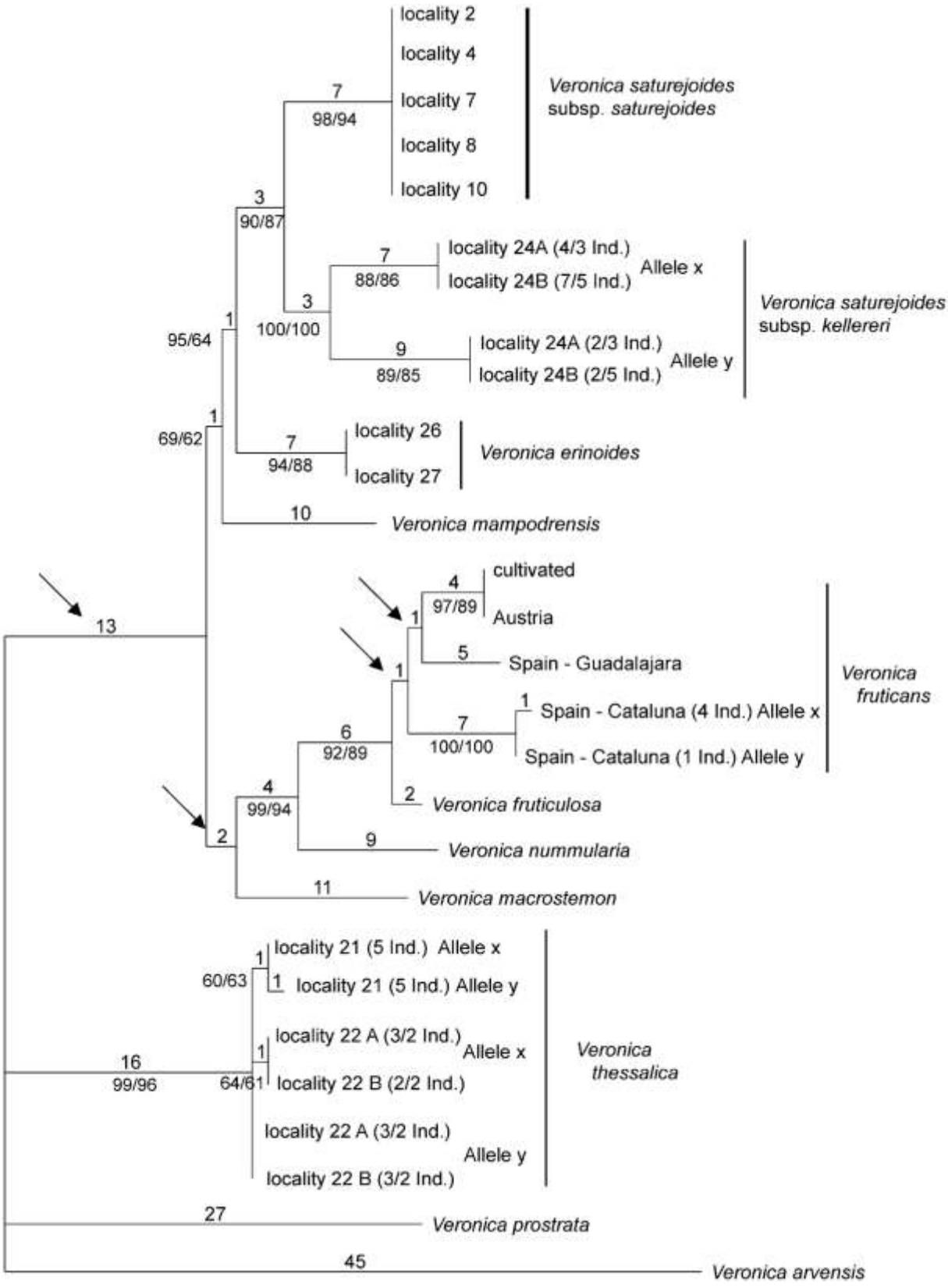
Table 3. Information on the four DNA datasets
* note that for the CHSi data set one sequence per population was used in the analysis unless the population was polymorphic. The number, thus, reflects neither the number of haplotypes

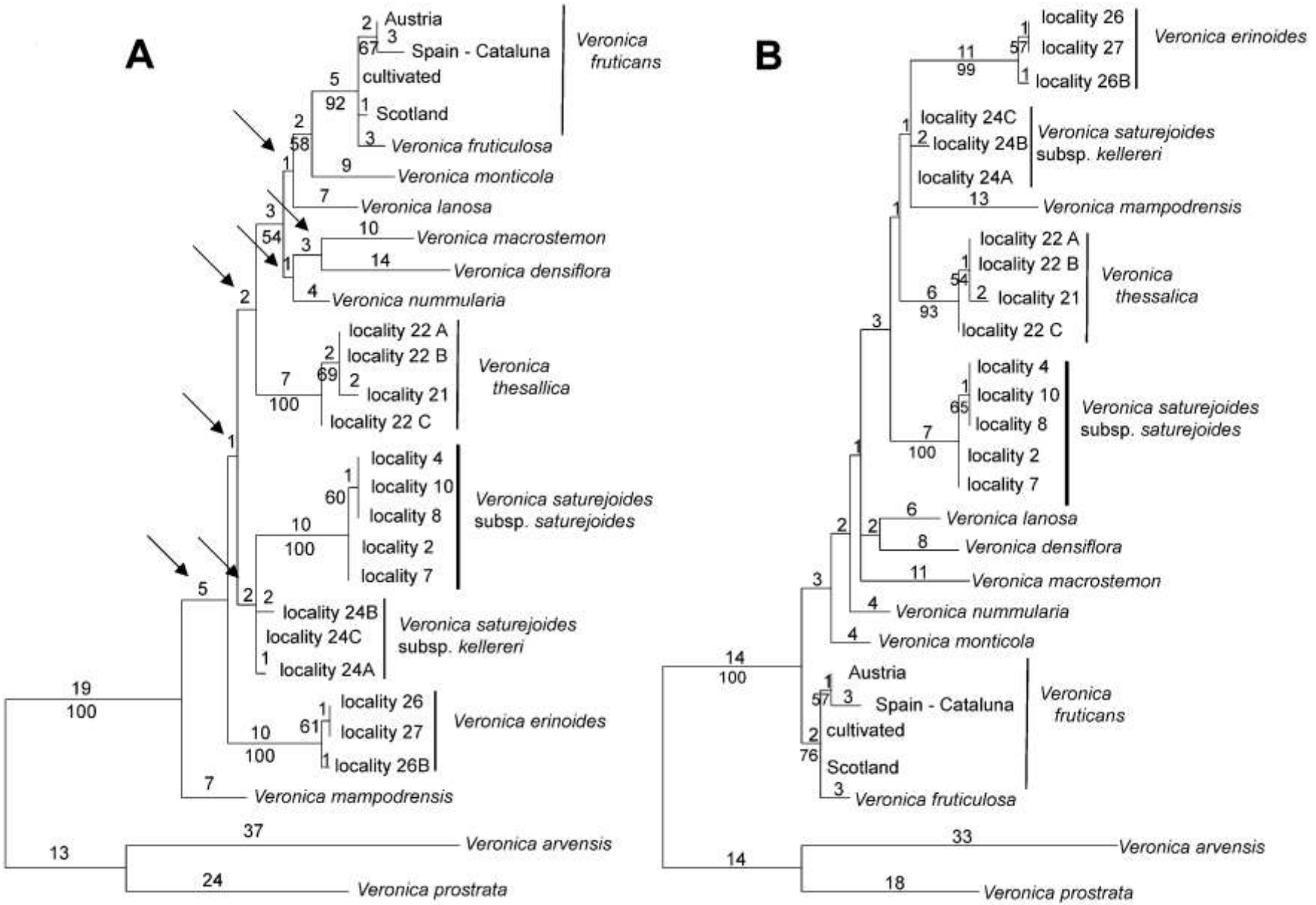
nor the number of sequences actually generated. CHSi = chalcone synthase intron; GC = guanine–cytosine; ITS = internal transcribed spacer.

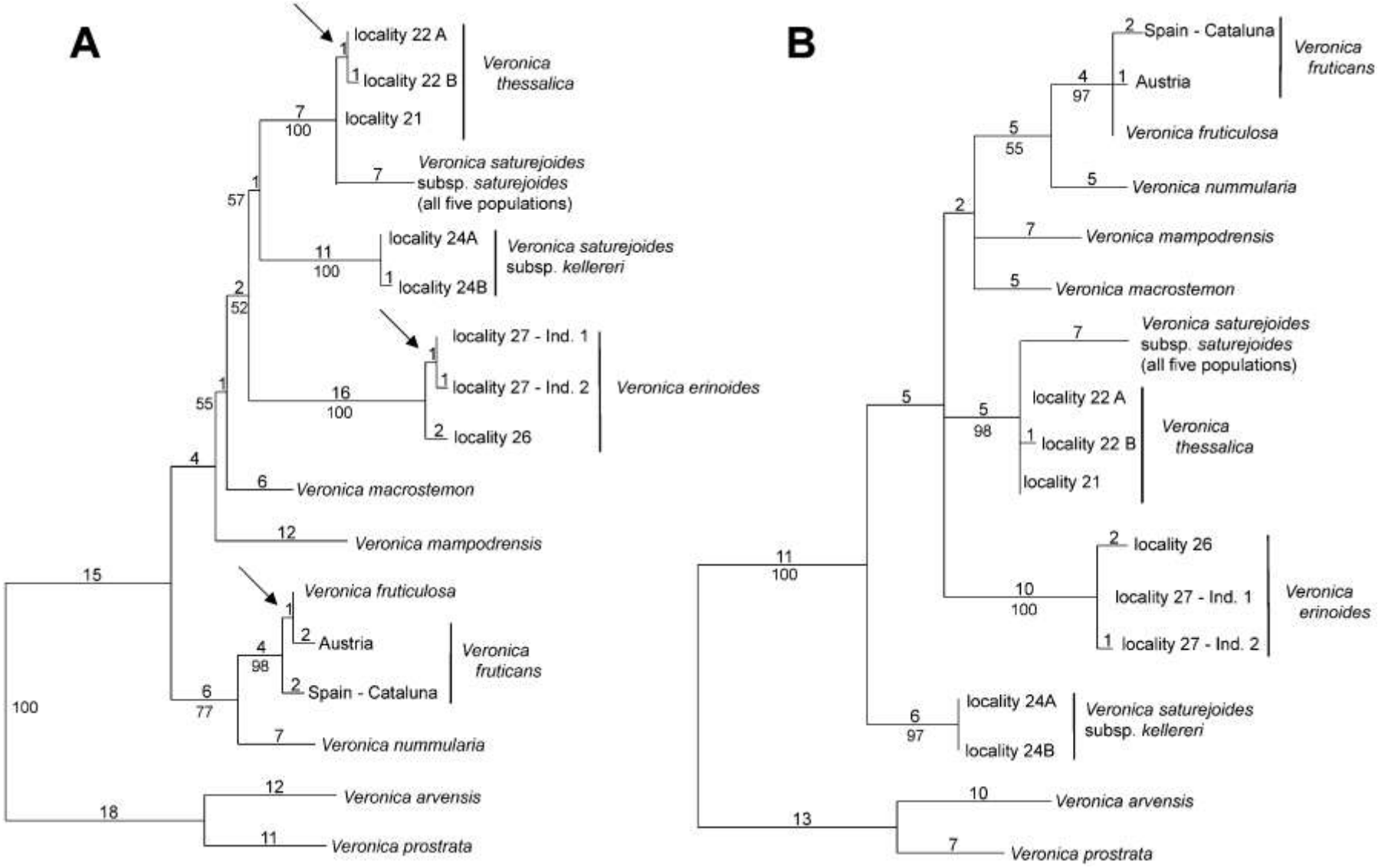
Table 4. Results of the Templeton tests

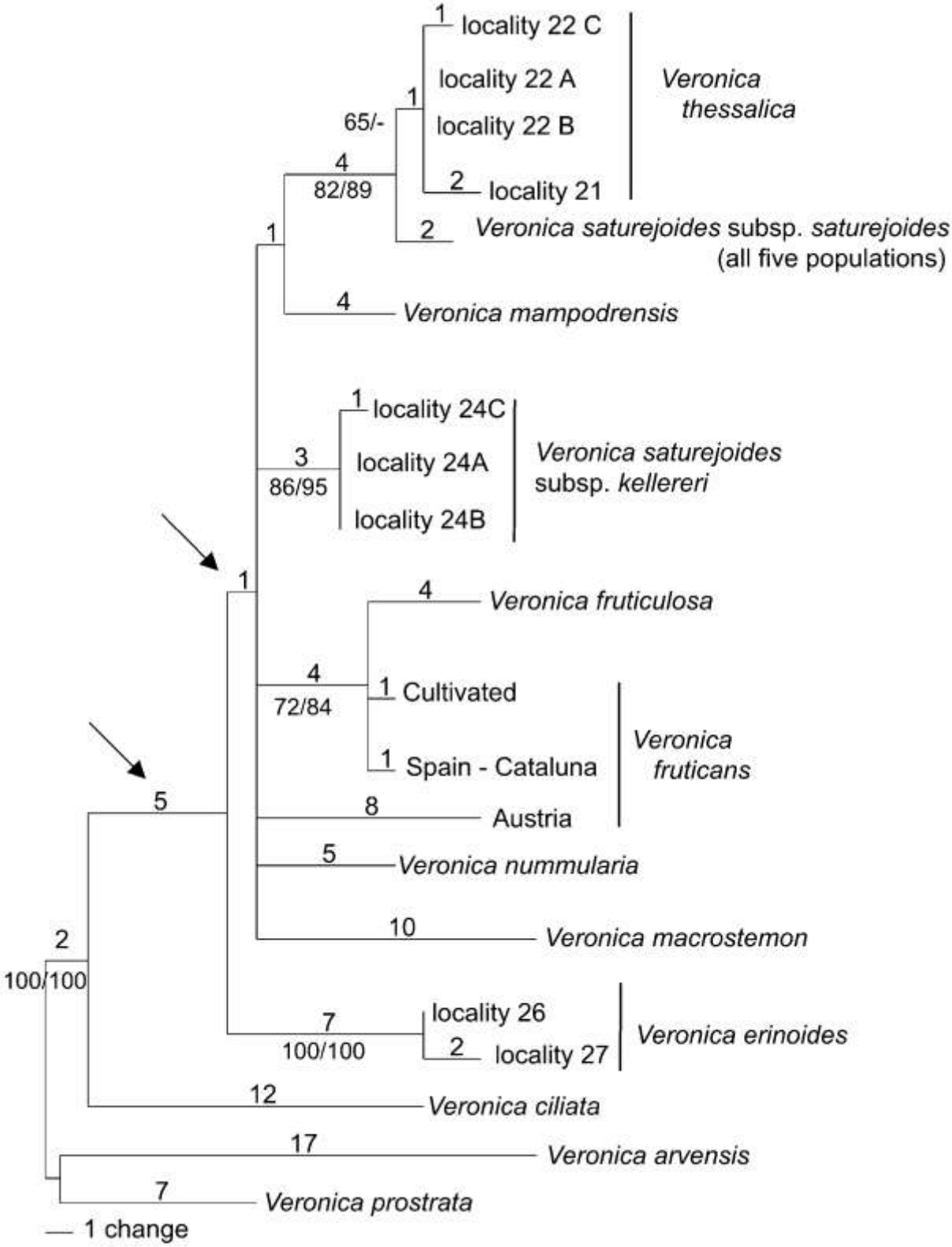
* significant tests (at 95%). CHSi = chalcone synthase intron; ITS = internal transcribed spacer; sat-the = test for the sister-group relationship of *V. saturejoides* subsp. *saturejoides* and *V. thessalica*; sat-kel = test for the monophyly of *V. saturejoides*.

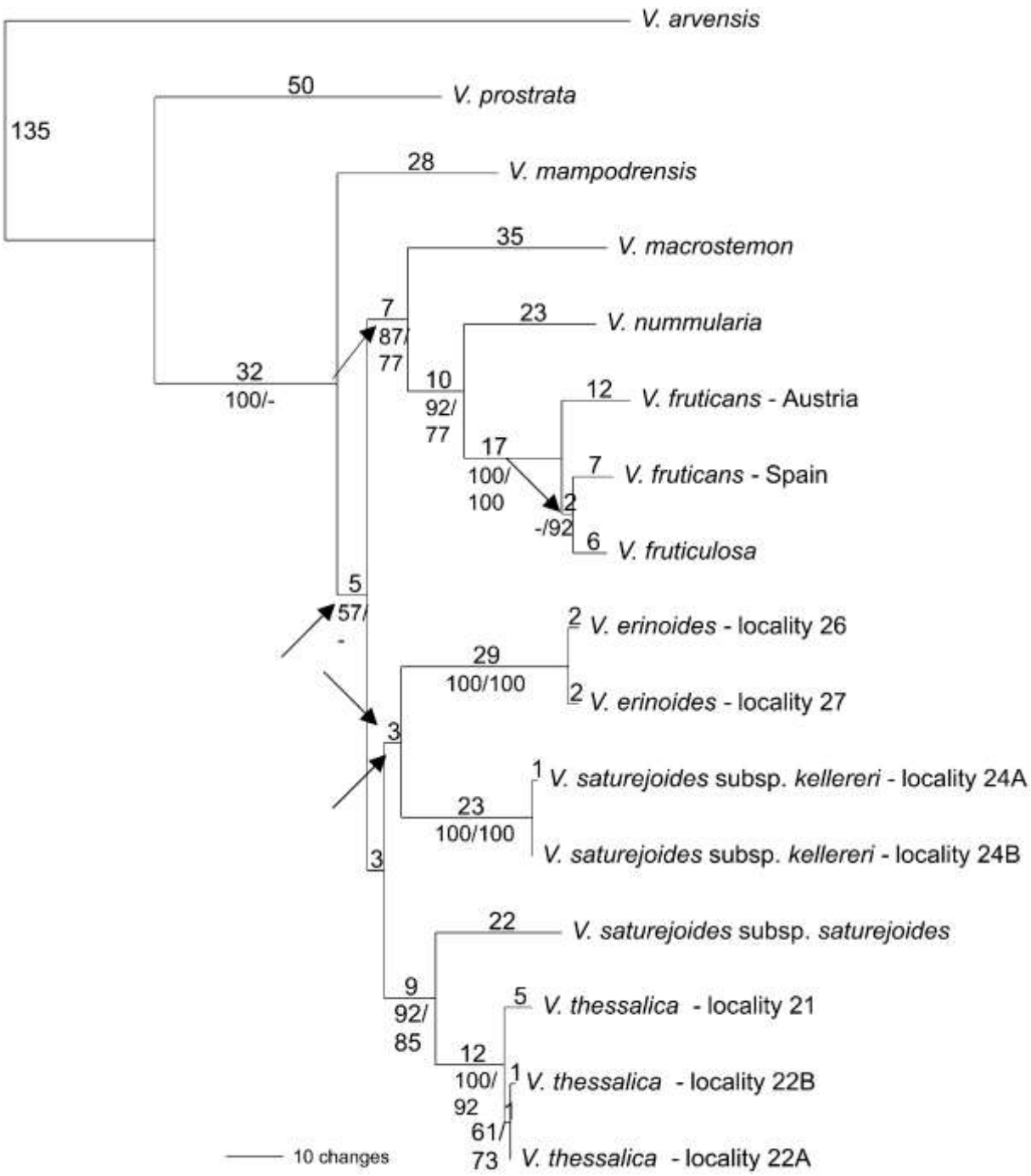

Croatia
Romania
Bosnia and Herzegovina
Serbia
Bulgaria
Montenegro
Italy
Albania
Macedonia
Greece
1
2*
3
4*
5
6
7*
8*
9
10*
11
12
13
14
15
16
17
18
19
20
21*
22*
23
24*
25**
26*
27*
28**
29**
30**
31
V. saturejoides ssp. saturejoides
V. saturejoides ssp. munellensis
V. saturejoides ssp. kellereri
V. thessalica
V. erinoides

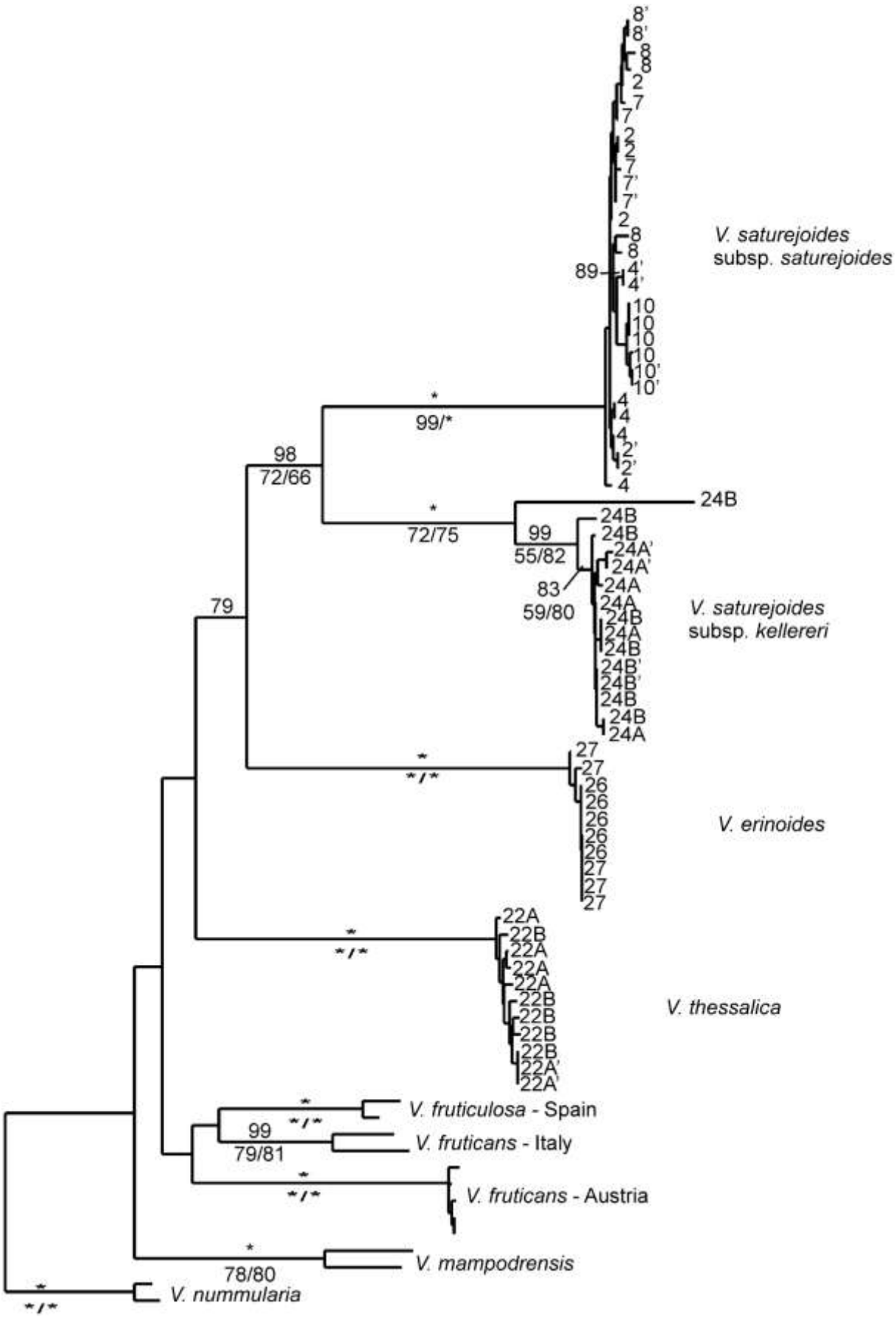

locality 2
locality 4
locality 7
locality 8
locality 10
Veronica saturejoides subsp. saturejoides
7
98/94
3
90/87
locality 24A (4/3 Ind.)
locality 24B (7/5 Ind.)
Allele x
7
88/86
3
100/100
1
95/64
locality 24A (2/3 Ind.)
locality 24B (2/5 Ind.)
Allele y
9
89/85
Veronica saturejoides subsp. kellereri
1
69/62
locality 26
locality 27
7
94/88
Veronica erinoides
10
Veronica mampodrensis
13
cultivated
4
97/89
Austria
1
5
Spain - Guadalajara
1
1
Spain - Cataluna (4 Ind.) Allele x
7
100/100
Spain - Cataluna (1 Ind.) Allele y
Veronica fruticans
6
92/89
2
Veronica fruticulosa
4
99/94
2
9
Veronica nummularia
11
Veronica macrostemon
1
locality 21 (5 Ind.) Allele x
60/63
1
locality 21 (5 Ind.) Allele y
1
locality 22 A (3/2 Ind.)
Allele x
16
99/96
64/61
locality 22 B (2/2 Ind.)
locality 22 A (3/2 Ind.)
Allele y
locality 22 B (3/2 Ind.)
Veronica thessalica
27
Veronica prostrata
45
Veronica arvensis

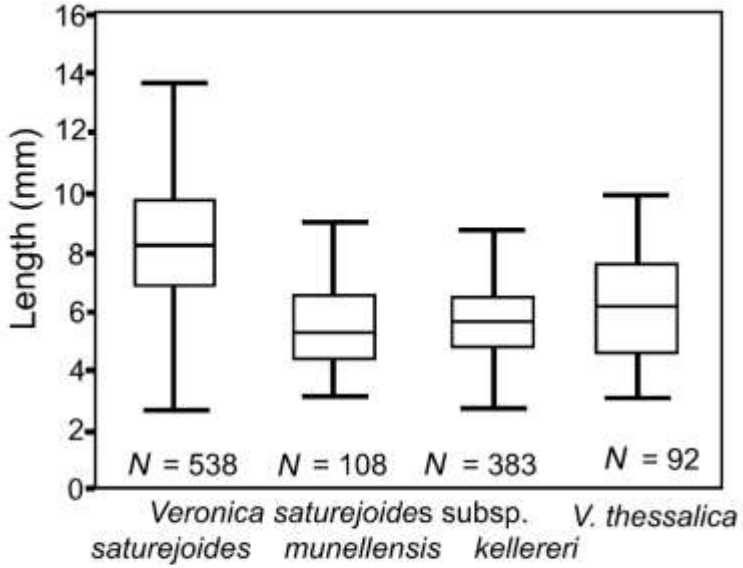

A
Austria
Spain - Cataluna
cultivated
Scotland
Veronica fruticans
Veronica fruticulosa
Veronica monticola
Veronica lanosa
Veronica macrostemon
Veronica densiflora
Veronica nummularia
locality 22 A
locality 22 B
locality 21
locality 22 C
Veronica thesallica
locality 4
locality 10
locality 8
locality 2
locality 7
Veronica saturejoides subsp. saturejoides
locality 24B
locality 24C
locality 24A
Veronica saturejoides subsp. kellereri
locality 26
locality 27
locality 26B
Veronica erinoides
Veronica mampodrensis
Veronica arvensis
Veronica prostrata
B
locality 26
locality 27
locality 26B
Veronica erinoides
locality 24C
locality 24B
locality 24A
Veronica saturejoides subsp. kellereri
Veronica mampodrensis
locality 22 A
locality 22 B
locality 21
locality 22 C
Veronica thessalica
locality 4
locality 10
locality 8
locality 2
locality 7
Veronica saturejoides subsp. saturejoides
Veronica lanosa
Veronica densiflora
Veronica macrostemon
Veronica nummularia
Veronica monticola
Austria
Spain - Cataluna
cultivated
Scotland
Veronica fruticans
Veronica fruticulosa
Veronica arvensis
Veronica prostrata

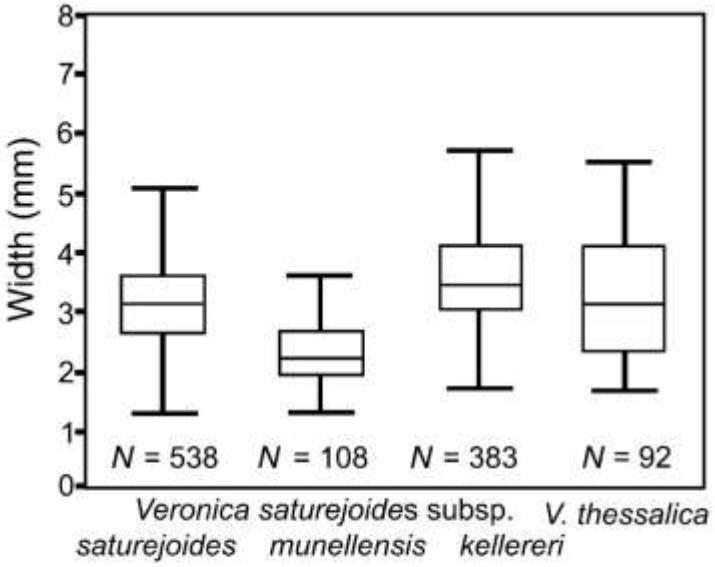

A
locality 22 A
locality 22 B
locality 21
Veronica thessalica
Veronica saturejoides subsp. saturejoides (all five populations)
locality 24A
locality 24B
Veronica saturejoides subsp. kellereri
locality 27 - Ind. 1
locality 27 - Ind. 2
locality 26
Veronica erinoides
Veronica macrostemon
Veronica mampodrensis
Veronica fruticulosa
Austria
Spain - Cataluna
Veronica fruticans
Veronica nummularia
Veronica arvensis
Veronica prostrata
B
Spain - Cataluna
Austria
Veronica fruticans
Veronica fruticulosa
Veronica nummularia
Veronica mampodrensis
Veronica macrostemon
Veronica saturejoides subsp. saturejoides (all five populations)
locality 22 A
locality 22 B
locality 21
Veronica thessalica
locality 26
locality 27 - Ind. 1
locality 27 - Ind. 2
Veronica erinoides
locality 24A
locality 24B
Veronica saturejoides subsp. kellereri
Veronica arvensis
Veronica prostrata

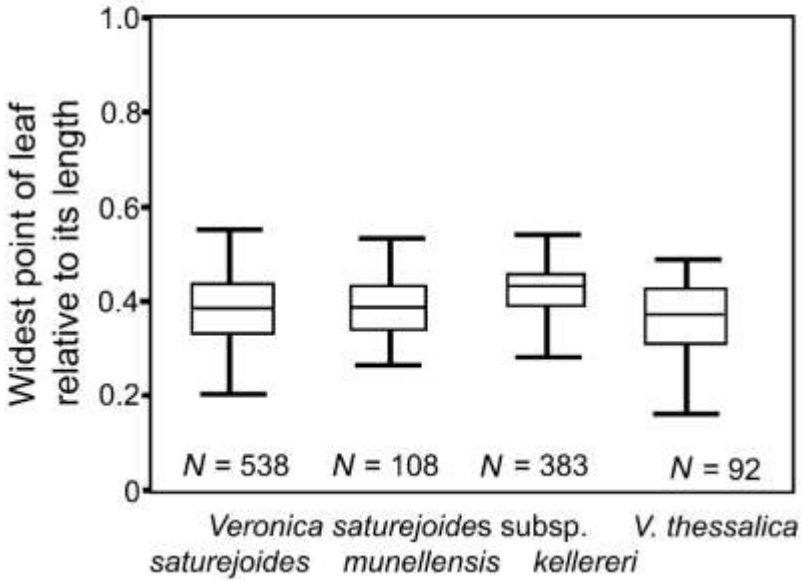

1
locality 22 C
locality 22 A
locality 22 B
2
locality 21
Veronica thessalica
1
65/-
4
82/89
2
Veronica saturejoides subsp. saturejoides
(all five populations)
1
4
Veronica mampodrensis
1
locality 24C
3
86/95
locality 24A
locality 24B
Veronica saturejoides
subsp. kellereri
1
4
Veronica fruticulosa
4
72/84
1
Cultivated
1
Spain - Cataluna
Veronica fruticans
5
8
Austria
5
Veronica nummularia
10
Veronica macrostemon
2
100/100
7
100/100
locality 26
2
locality 27
Veronica erinoides
12
Veronica ciliata
17
Veronica arvensis
7
Veronica prostrata
1 change

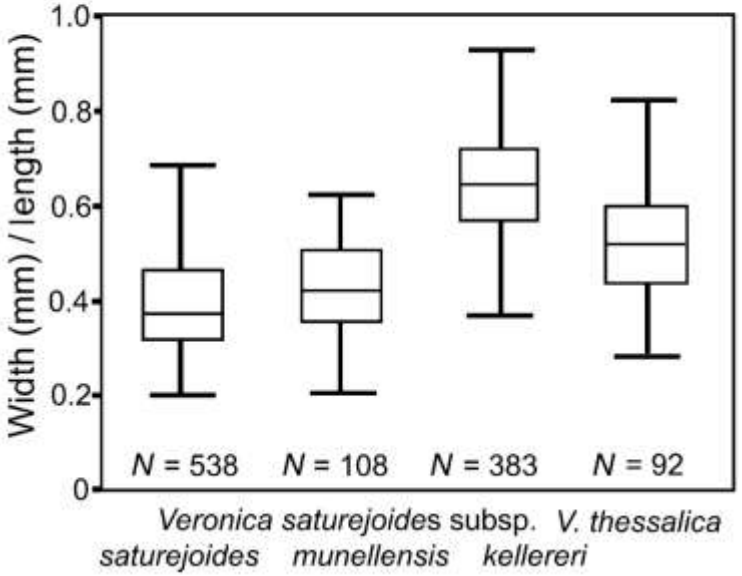

V. arvensis
50
V. prostrata
135
28
V. mampodrensis
35
V. macrostemon
7
87/
77
23
V. nummularia
32
100/-
10
92/
77
12
V. fruticans - Austria
17
100/
100
7
V. fruticans - Spain
2
-/92
6
V. fruticulosa
5
57/
-
2
V. erinoides - locality 26
29
100/100
2
V. erinoides - locality 27
3
1
V. saturejoides subsp. kellereri - locality 24A
23
100/100
V. saturejoides subsp. kellereri - locality 24B
3
22
V. saturejoides subsp. saturejoides
9
92/
85
5
V. thessalica - locality 21
12
100/
92
1
V. thessalica - locality 22B
1
61/
73
V. thessalica - locality 22A
10 changes


V. saturejoides subsp. saturejoides
V. saturejoides subsp. kellereri
V. erinoides
V. thessalica
V. fruticulosa - Spain
V. fruticans - Italy
V. fruticans - Austria
V. mampodrensis
V. nummularia
89
99/*
98
72/66
72/75
99
55/82
83
59/80
79
78/80
99
79/81


Length (mm)
16
14
12
10
8
6
4
2
0
N = 538
N = 108
N = 383
N = 92
Veronica saturejoides subsp.
saturejoides
munellensis
kellereri
V. thessalica


Width (mm)
8
7
6
5
4
3
2
1
0
N = 538
N = 108
N = 383
N = 92
Veronica saturejoides subsp.
saturejoides
munellensis
kellereri
V. thessalica


Widest point of leaf
relative to its length
1.0
0.8
0.6
0.4
0.2
0
N = 538
N = 108
N = 383
N = 92
Veronica saturejoides subsp.
saturejoides
munellensis
kellereri
V. thessalica


Width (mm) / length (mm)
1.0
0.8
0.6
0.4
0.2
0
N = 538
N = 108
N = 383
N = 92
Veronica saturejoides subsp.
saturejoides
munellensis
kellereri
V. thessalica

Species	Country	Locality	No. of Individuals	GenBank accession numbers				Voucher
				CHSi	ITS	*rpoB-trnC*	*trnL-trnL-trnF*	
Veronica ciliata	China	Qinghai	1	–	–	–	AF486385	Miehe *et al.* 98-33313, GOET
V. densiflora	Russia	Altai	1	–	AY741521	–	–	M. Staudinger s.n., SALA
V. erinoides	Greece	26	1	–	AY741523	–	–	Hagemann, Scholz & Schmitz 461, SALA
V. erinoides	Greece	26	10	**EU282055**	**EU282103**	**EU282066**	**EU282091**	M. von Sternburg 022, WU
V. erinoides	Greece	27	9	**EU282054**	**EU282102**	**EU282068**	**EU282092**	M. von Sternburg 018, WU
V. fruticans	Austria	Carinthia, Hohe Tauern: Goldberggruppe	5	**EU282056**	**EU282107**	**EU282073**	**EU282083**	P. Schönswetter 7.7.2006, WU
V. fruticans	Spain	Cataluña, Lleida: Aiguestortes.	4	**EU282057** EU282058	**EU282108**	**EU282071**	**EU282084**	P. Schönswetter & B. Frajman 3.8.2006, WU
V. fruticans	Spain	Guadalajara, El Cardoso de la Sierra, Pico del Lobo	1	EU282059	–	–	–	M. M. Martínez-Ortega 1009, SALA
V. fruticans	Great Britain	Scotland	1	–	AY144462	–	–	V. Halcro 30, K
V. fruticulosa	Cultivated	New York Botanical Garden	1	–	AF313005	–	AF486393	L. Struwe 1408, WU
V. fruticulosa	Cultivated	Botanical Garden Bonn	1	**EU282060**	**AF313004**	**EU282076**	**AF486383**	D. C. Albach 71, BONN
V. lanosa	Pakistan	–	1	–	AY540868	–	–	Schickhoff 1377, GOET
V. macrostemon	Russia	Altai	1	**EU282063**	**AY741522**	**EU282082**	**AY486441**	M. Staudinger AL23-18, SALA
V. mampodrensis	Spain	Palencia	1	**EU282062**	**DQ227331**	**EU282077**	**DQ227337**	M. M. Martinez-Ortega *et al.* 713, SALA
V. monticola	Georgia	–	1	–	DQ227333 DQ227334	–	–	Iranisvili 26071983, WU
V. nummularia	Spain	Cataluña, Gerona: Tosses, Niu d'Aliga	1	**EU282061**	**DQ227335**	**EU282081**	**EU282094**	M. M. Martínez-Ortega & L. Delago 718, SALA
V. saturejoides subsp. *kellereri*	Bulgaria	24 – Mt Vihren A	9	**EU282040** EU282041	**EU282096**	**EU282075**	**EU282086**	P. Schönswetter & B. Frajman 28.06.2006, WU
V. saturejoides subsp. *kellereri*	Bulgaria	24 – Mt Vihren B	14	**EU282038** EU282039	**EU282095**	**EU28270**	**EU282085**	M. von Sternburg 29.06.2006, WU
V. saturejoides subsp. *kellereri*	Bulgaria	24	1	–	AY144461	–	AY486450	D. C. Albach 558, WU

V. saturejoides subsp. *saturejoides*	Bosnia-Herzegovina	4	5	**EU282042**	**EU282097**	**EU282072**	**EU282087**	B. Surina 19.07.2006, WU
V. saturejoides subsp. *saturejoides*	Bosnia-Herzegovina	2	3	EU282045	EU282100	Identical to above	Identical to above	B. Surina 18.07.2006, WU
V. saturejoides subsp. *saturejoides*	Bosnia-Herzegovina	7	5	EU282044	EU282099	Identical to above	Identical to above	B. Surina 20.07.2006, WU
V. saturejoides subsp. *saturejoides*	Bosnia-Herzegovina	8	10	EU282046	EU282101	Identical to above	Identical to above	B. Surina 05.07.2006, WU
V. saturejoides subsp. *saturejoides*	Bosnia-Herzegovina	10	5	EU282043	EU282098	Identical to above	Identical to above	B. Surina 22.07.2006, WU
V. saturejoides subsp. *saturejoides*	Bosnia-Herzegovina	12	4	Identical to above	Identical to above	FJ620683	Identical to above	B. Surina 9.7.2008, NHM Rijeka
V. thessalica	Macedonia	21	10	**EU282052** EU282053	**EU282106**	**EU282079**	**EU282088**	P. Schönswetter & B. Frajman 15.08.2006, WU
V. thessalica	Greece	22A	8	**EU282048** EU282049	**EU282074**	**EU282074**	**EU282090**	M. von Sternburg 27.07.2006, WU
V. thessalica	Greece	22B	5	**EU282050** EU282051	**EU282105**	**EU282067**	**EU282089**	P. Schönswetter & B. Frajman 17.08.2006, WU
V. thessalica	Greece	22C	1	–	AF509792	–	AF513343	Raus & Rogl 5072, SALA
V. arvensis	Germany	Stromberg bei Bockenau		**EU282065**	**AF313002**	**EU282078**	**AF486380**	D. C. Albach 147, WU
V. prostrata	Austria	–		**EU282064**	–	**EU282080**	**EU282093**	D. C. Albach 860, MZJG
V. prostrata	Cultivated	Botanical Garden, Bonn		–	**EU282109**	–	–	D. C. Albach 67, BONN

V. saturejoides* subsp. *saturejoides

Cultivated material

Edinburgh (Cult.) 04-05.1896; K H2006/01047-7 & E E32542

Bosnia and Hercegovina

1 – Mount Vitorog 44°45' N; 16°27' E: 1600 m; J. Stadlmann & F. Faltis 18.07.1904; WU 2242

2 – Šator Planina 44°9’ N; 16°36’ E: 1680 - 1840 m; E. Janchen 16.07.1904; WU 2436 / 1872 m; B. Surina 18.07.2006; WU

3 – Dinaric Mountains, Mount Dinara 44°3’ N; 16°23’; E: unknown collector; E E32543 / 1680 m; I. Horvat 18.07.1930; WU

4 – Dinaric Mountains, Mount Troglav 43°56’ N; 16°35’ E: 1500 - 1600 m; E. Janchen & B. Watzel 04.07.1907; WU 2375 / 1900 m; B. Surina 19.07.2006; WU

7 – Dinaric Mountains, Mount Cincar 43°54’ N; 17°3’ E: 1600 - 2 m; F. Fiala 20.06.1893; B B100217748 / 1400 m; B. Surina 20.07.2006; WU

8 – Vran Planina 43°40’ N; 17°29’ E: J. Stadlmann, F. Faltis, E. Wibiral 25.07.1907; WU 2754

9 – Bjelašnica Mountains, Mount Hranisava 43°44’ N; 18°8’ E: 1800 m; G. Beck 06.1888; K H2006/01047-7 & EE32544 & GG86663 & W 3758, 5714, 15915 & B B100217746 & WU 1467, 1788 / K. Maly 18.06.1905; B B100217750 / Curcic 01.06.1898; W 8926 / Sagorski 18.06.1906; W 3607 / 1800 m; G. & M. A. Fischer 29.07.1972; WU

10 – Mount Velika Velež 43°17'36,8" N 18°4'44,3" E: 1640 m, B. Surina 22.07.2006, WU

11 – Lelija Planina 43°25’ N; 18°29’ E: J. A. Knapp 1869; G:G86656

Croatia

Unknown locality: K. Maly; B: B100217751 & G:G86655 & W

3 – Dinaric Mountains, Mount Dinara 44°3’ N; 16°23’ E: K. Maly; W 11309

5 – Dinaric Mountains, Mount Prologh 43°50’ N; 16°41’ E: 1200 m; T. Pichler 18.07.1868; BM 68744, G 86660, 86664, 86666, W, WU 2804 / T. Pichler 07.1872; WU 1788

6 - Dinaric Mountains, Mount Kamešnica 43°45’ N; 16°48’ E: T. Pichler 06.1872; B B100217742, BM 68745, G G86654, G86662, G86665, K H2006/01047-2, W 15968, WU s.n., WU 2804 / 1500 m; T. Pichler 07.1870; G G86661, K H2006/01047-3, W 8682, WU s.n., WU 771 / 1200 - 1600 m; H. Handel-Mazzetti 04.07.1909; WU 2436

Montenegro

Unknown locality: W. Dod; BM 68742

12 – Mount Durmitor 43°8’ N; 19°2’ E: A. Baldacci 08.1890; G G86657, W 5176, WU 1169

13 – Mount Sinjavina 42°57’ N; 19°20’ E: A. Baldacci 21.08.1891; G 86658, K H2006/01047-5

14 – Mount Gradište, near Kolašin 42°52’ N; 19°21’ E: J. Rohlena 07.1903; Z 39140

V. saturejoides* subsp. *munellensis

Albania

15 – Mount Munella 41°58’ N; 20°6’ E: A. Baldacci 30.06.1897; K H2006/01047-7, G 86659

16 – Mount Deja; 41°43’ N; 20°9’ E: 2100 m; Guiseppi; K H2006/01047-6 23

V. saturejoides* subsp. *kellereri

Bulgaria

23 – Rila Mountains, area of Ribni jesera 42°7’ N; 23°29’ E: W. Hilbig 06.08.1978; HAL 067565

24 – Pirin Mountains

Unknown locality: Kellerer 1909; SOM 67911 / M. Antsev 19.08.1973; SOM 125269 / 1880 m; N. Andreev 15.06.1976; SOM 134175 / R. Taskova 05.07.1996; SOM: 153318

Kazana 41°46'12" N; 23°24'37" E: 2100 m; B. Kuzmanov 21.07.1980; B B100217745, G 86667 / 2380 m; B. Acktarov 07.08.1938; SOM 67913 / 2400 m; B. Acktarov 11.08.1938; SOM: 67915, 67917 / 2400 m; B. Kuzmanov 14.07.1972; SOM 128053 / 2500 m; B. Kuzmanov 10.08.1976; G 86668 / 2350 m; J. Röthlisberger 13.07.2001; Z 39139/ 2100-2900m; B.

Frajman & P. Schönswetter 28.6.2006, WU / 2290m; M. von Sternburg 29.6.2006, WU / 2293 m; M. von Sternburg 2.7.2006, WU / 2302 m; M. von Sternburg, 2.7.2006, WU

Mount Bajuvi Dupki: Kellerer 05.07.1924; SOM 67909 / B. Acktarov 09.08.1939; SOM 67916

Mount Koncheto 41°47'34" N; 23°22'50" E: 2500 m;; F. Cernoch 17.07.1967; B 100217743

Mount Pirin 41°40' N; 23°30' E: J. K. Urumov 20.07.1915; SOM 67822, 67920

Mount Vihren 41°46' N; 23°24' E: Rev. & Mrs. H. P. Thompson 28.07.1933; K H2006/01047-1 / I. Horvat 24.08.1936; WU / G. Beck 20.08.1976; B100217744 / 2300 m; B. Acktarov 12.08.1938; SOM 67919 / 2600 m; B. Kitanov 10.07.1940; SOM 96252 / E. Jaeger 30.07.1961; HAL 074703 / D. Peev 18.07.1964; SOM 124413 / 2500- 2700 m; unknown collector 07.1967 G 86669 / 2400 - 2700 m; H. Seitter 1967; ZT 12698 / 2200 m and 2914 m; G. & M. A. Fischer 27.07.1971; WU (six sheets) / N. Andreev 21.07.1973; SOM 134147 / 2200 m; R. Taskova 06.07.1996; SOM 153319 / 1965 m; D. Albach 22.06.2001; WU 4478/025663 / 2387 m, M. von Sternburg 29.6.2006, WU / 2322 m; M. von Sternburg 2.7.2006, WU

Okadenski Rid 41°48'33" N; 23°21'40" E: 2150 m; 24.07.1952; D. Jordanov & B. Kitanov; HAL 09350, SOM 67677, W 1953/4232

Razloski sycholol 41°47'7" N; 23°23'30" E: D. Peev 26.08.1964; SOM 128937 / 2040 m; V. Goranova 23.07.2004; SOM 161693 / 2200 m; S. Tsoneva 23.07.2004; W 2005 / 5929

V. thessalica

Albania

18 – Luma district, Galica Lums (=Djalica-e-Lumes) 42°0'57" N; 20°28'18" E: 2470 m; I. Dörfler 19.06.1918; B 100311205, C 52/2006, BM 67963, G 86670, W 1927/9372, ZT 12699 / 2200-2400 m; H. Zerny 19.06.1918; W 1958 / 25178

Macedonia

19 – Ljuboten, Mount Skardus 42°12'22" N; 21°6'20" E: J. Bornmüller 26.07.1918; B 100217747 / 2450 m; Rev. & Mrs. H. P. Thompson 17.06.1937; K H2006/01047-4

20 – Golsnica planina, Mount Pepeljak 41°48' N; 21°20' E: 2250-2300 m; J. Bornmüller 21.06.1918; B 100311204

21 – Mount Jakupica 41°43´16" N; 21°24´50" E: B. Frajman & P. Schönswetter 15.8.2006, WU

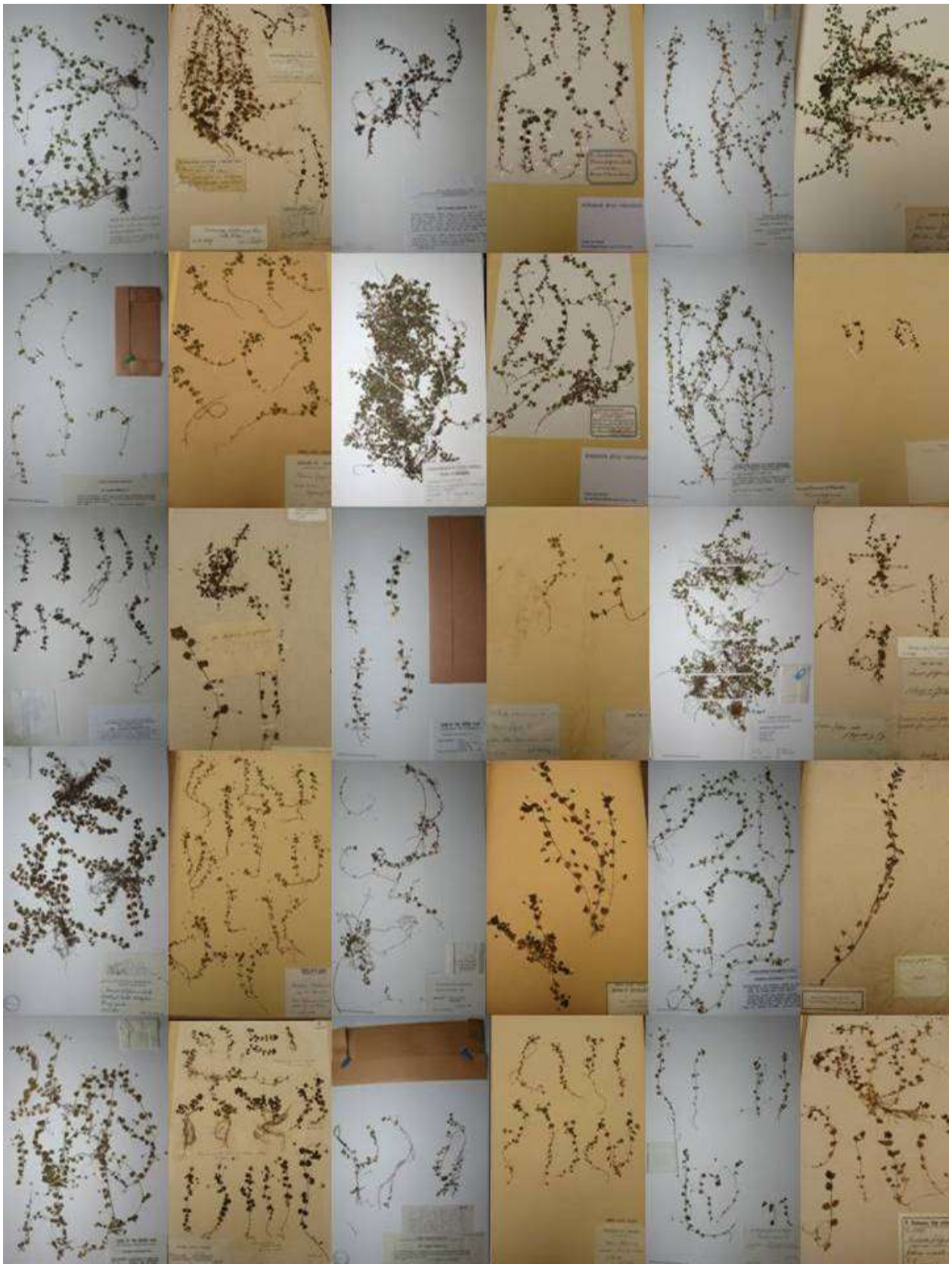
Greece

22 – Macedonia region, Prefecture Pieria, Mount Olimbos 40°5'0" N; 22°21'0" E: 2850 m; H. Handel-Mazzetti 15.07.1927; W 1927/19098 / 2850 m; O. Dibowski 07.07.1928; G 86678, W 1928/7018 / 2800 m; Metlesics 03.06.1958; W 1960/17730 / 2800-2850 m; Gneuser 26.07.1971; G 86677 / 2100m; Klaus, Kummert & Mück 07.06.1972; W 1973/01855 / 2700 m; A. Charpin & J. J. Lazare 19.09.1989; G 86680 / 2700m; M. von Sternburg 27.7.2006, WU / B. Frajman & P. Schönswetter 17.8.2006, WU

	CHSi	ITS	Combined nuclear	*rpoB-trnC*	*trnL-trnL-trnF*	Combined plastid	Combined
Number of sequences in separate analyses	28*	29	16	17	26	16	16
Average GC content	35.0%	56.6%	–	30.8%	35.0%	–	–
Characters in the alignment with/without outgroup	1148/649	768/763	1916/1412	1296/1262	925/924	2221/2186	4137/3598
Scored indels	16	28	44	32	16	48	92
Parsimony informative characters	49	97	80	44	52	57	137
Number of most-parsimonious trees	65	405	2	12	10562	12	6
Tree length of most-parsimonious trees	179	215	289	152	134	187	482
Consistency index	0.97	0.81	0.73	0.96	0.87	0.90	0.77
Retention index	0.97	0.87	0.80	0.94	0.88	0.93	0.82
Substitution model in maximum likelihood analysis	TrN+I	GTR+I+Γ	–	TVM+Γ	TVM+I	–	GTR+Γ
Number of maximum likelihood trees	1	2	–	1	1	–	
ln L of maximum likelihood tree	2340.00	2066.49	–	2398.77	1853.30	–	

Topology →	CHSi	ITS	*rpoB-trnC*	*trnL-trnL-trnF*	sat-the	sat-kel
CHSi	–	<0.04*	<0.04*	<0.04*	0.02*	–
ITS	<0.001*	–	<0.001*	<0.24	0.52	–
rpoB-trnC	<0.003*	<0.04*	–	<0.48	–	0.01*
trnL-trnL-trnF	<0.04*	<0.03*	<0.31	–	–	0.18

“DIVERSITY OF HERBARIUM SHEET IN *VERONICA FILIFORMIS*”



4 DEGRADATION OF SEXUAL REPRODUCTION

"Degradation of sexual reproduction in* Veronica filiformis *after introduction to Europe"

by R. Scalone[1] and D. Albach[2, §]

from:

[1] Institut für Spezielle Botanik und Botanischer Garten, Johannes Gutenberg-Universität Mainz, Germany

[2] Institut für Biologie und Umweltwissenschaften (IBU), Carl von Ossietzky-Universität Oldenburg, Germany

[§] author for correspondence: D. Albach, Institut für Biologie und Umweltwissenchaften (IBU), Carl von Ossietzky Universität, Carl von Ossietzky-str. 9-11, 26111 Oldenburg, Germany.

Email: dirk.albach@uni-oldenburg.de,

Phone: (0049) - 441 798 3339,

Fax: (0049) - 441 798 3331,

Keywords: Crossing experiment; AFLP; pollen & ovule productions; seed investigations; Muller´s ratchet; invasion; *Veronica.*

Running head: Reproductive biology of the self-incompatible weed, *Veronica filiformis*

4.1 INTRODUCTION

Mankind is changing its environment in hitherto unprecedented ways. One aspect of change is the introduction of organisms to a new environment, thus creating many evolutionary "experiments". While most of these introductions fail (Blackburn *et al.*, 2011), those successful ones are often the source of economic and ecological problems (Pimentel *et al.*, 2000). However, they can also be important to understand processes of evolution. In particular, mating system evolution of newly founded populations is a highly relevant aspect of introductions (Baker, 1974; Rambuda & Johnson, 2004). Based on observations of plant species introduced to islands, Baker (1955) concluded that these species are more likely to be self-compatible (SC) and capable of autonomous self-pollination. This capacity is an advantage during the establishment of populations from a single or very few individuals in a new environment where encountering a mating partner or an efficient pollinator is doubtful. This observation has subsequently been termed "Baker´s rule" (Baker, 1967; Stebbins, 1957). The maintenance of a self-incompatibility (SI) system in a natural population requires the presence of a large number of S-alleles in each population (~30-40; Lawrence, 1996). This point is critical for the introduction and for the survival of populations of self-incompatible

species in a new environment. Usually, an introduced population is founded by few individuals that are genetically closely related and therefore share a low and limited number of alleles. In that case, after the first generations, all individuals belonging to the new population likely share the same S-alleles after inbreeding and sexual reproduction becomes impossible (Byers & Meagher, 1992).

However, Baker's rule can be avoided in two different ways: the breakdown of SI or a switch to vegetative reproduction. The SI species can establish itself either by selection for selfing and disruption of their SI system or by reallocation of their resources from sexual to vegetative reproduction (Barrett *et al.,* 1989; Eckert, 2002). Both strategies have essentially the consequence that genetic variation decreases and adaptation to the new environment is impaired ("evolutionary dead-end", Stebbins, 1957; Ornduff, 1969). Moreover, further reduction of genetic diversity occurs in bottlenecks caused by small population size of the initial colonizing population ("founder effect"). Finally, this small initial introduced population will be subject to the Allee effect, the low success of outcrossing at low density, and possibly drives the population to extinction (Lamont *et al.,* 1993; Davis *et al.,* 2004; Courchamp *et al.,* 2008). Therefore, introduced selfing or clonal populations are expected to have especially low genetic variation (Takebayashi & Morrell, 2001). The necessity of high genetic diversity in invasive plants has been a matter of debate for some time (Hollingworth & Bailey, 2000; Grimsby *et al.,* 2007; Novak, 2007) and apparently, genetic diversity does not need to be high in introduced populations of organism to be successful invaders (Poulin *et al.,* 2005; Culley & Hardiman, 2009). In the extreme case, a single clone of *Fallopia japonica* has been shown to invade all Britain and probably the whole of Europe (Hollingsworth & Bailey, 2000, 2008). Such a successful genotype can either be narrowly pre-adapted to the environment encountered or constitute a "general-purpose genotype" sensu Baker (1955) being able to react to the environment in a way that assures high fitness. Nevertheless, more and more studies have detected large genetic variation even in clonal and invasive plants (Gabrielsen & Brochmann, 1998; Li & Ge, 2001). Increasing genetic diversity after the first introduction can be achieved either through hybridization with local relatives (Bleeker, 2003; Tiébré *et al.,* 2007) or through multiple introductions of the invasive species (Genton *et al.,* 2005). Even if genetic diversity is increased by mutations, this is likely to be predominantly deleterious in selfing and clonal species (Glémin *et al.,* 2006; Wright *et al.,* 2006) and the lack of sexual reproduction leads to their accumulation and further loss of fitness ("Muller´s ratchet"; Rottenberg & Parker, 2004). Understanding patterns of genetic variation in

introduced plants, therefore, allows inferences of the biological factors influencing its spread and its future invasion potential (Poulin *et al*,. 2005).

We study these questions here in the Pontic-Anatolian-Caucasian *Veronica filiformis* (Figure 1), an obligate self-incompatible (SI) and perennial species (Lehmann, 1942, 1944; Lehmann & Schmitz-Lohner, 1954). The first records of *V. filiformis* came from Great Britain (1780, 1838) but it was not recorded again until 1927 on the island (Bangerter & Kent 1957, 1962, 1965). However on the continent, the first record (1893) in southern France (Marseille) is likely due to plants being packed around the roots of vine shoots imported from Georgia (Lehmann, 1909). From that time, the history of the introduction in the rest of Old World is fairly well-known starting in Switzerland (Geneve 1903), France (Haute-Saône 1904; Mayenne 1923; Rennes 1937), Germany (Tübingen 1909; München 1923; Ulm 1936, Augsburg 1939), Great Britain (Whisley 1927), Austria (Salzburg 1934; Wien 1941), the Netherlands (Tubergen-Haarlem 1936) and then other regions of Europe (Lehmann, 1909, 1942; Thaler, 1953; Bangerter & Kent, 1962; Figure 1). Most European populations of this species are considered and described as sterile in the different European floras because no seed production was observed since the apparition of this species in Old World (Lehmann, 1942 & 1944). This species may therefore offer a suitable subject to the study of consequences of an SI system during the colonization of Europe and the impact of the absence of sexual reproduction.

For these reasons, the objectives of this study were (1) to determine the reason(s) for the absence of seed production in Europe, (2) to explain the spread of the species by a putative clonal dispersal and finally, (3) to evaluate the impact of this colonization of Europe on the reproductive biology of *V. filiformis*. For this purpose, intra- and inter-population crossing experiments were conducted along a regional transect of twenty German populations (~150 km) in the area of first introductions in southern Germany (between Tübingen and Augsburg; T-U-A in Figure 1), followed by AFLP-fingerprinting of these German populations and native populations from Georgia and Turkey (green crosses; Figure 1). These experiments conducted in the greenhouse and the laboratory permitted to define crossing groups (i.e., one crossing group is composed of individuals which never produce any seeds when crossed with each other and who are, therefore, inferred to have the same SI alleles) and clones (i. e., two specimens are considered as clones when their rate of pairwise individual comparisons is below the error rate of the AFLP analysis) as well as to estimate the mate availability within these two German regions (Baden-Württemberg “BW” and Bavaria “BV”). Flower number

and morphology, pollen and ovule quantities, seed number and size, germination rate and seed viability, including embryo size, were measured on some pertinent populations from the introduced and native areas to determine the impact of lacking sexual reproduction on the reproductive biology of *V. filiformis*.

4.2 MATERIALS AND METHODS

4.2.1 Plant material

Between March and April 2007, eighteen populations of *Veronica filiformis* were collected along a transect between Tübingen (Baden-Württemberg), Ulm (Baden-Württemberg) and Augsburg (Bavaria) with a maximal distance of 150 km (T-U-A; Figure 1). The sampling was guided by the first observations in the region based on records by Lehmann (1942) and Thaler (1953) in "Tübingen Lustnau" (1909), old cemetery of Ulm at "Wiblingen" (1936), and unlocated public parks of "Augsburg" (1939). Since three populations ("Tubingen Lustnau" Tl – "Pliezhausen" Pl – "Reutlingen" Re) separated by distances between four (minimal) and ten kilometers (maximal) showed seed production during crossing experiments in 2007, two supplementary populations ("Kirchentellinsfurt" Kt – "Betzingen" Bz) were collected within the triangle formed by these populations. Thus, twenty populations were transplanted in 2008. Seventeen were crossed in 2007 and nineteen in 2008. Indeed, one of them, "Großaitingen" Gß, never produced flowers in the greenhouse and no flower was observed in the field during the two collecting trips (Table 1 and Figure 2 & 3).

Two Georgian populations, one in the Greater Caucasus ("Kazbegi-Kobi", Kz9) and one in the Lesser Caucasus ("Bakuriani-Tskratskaro", Bk10) were collected in June 2008 and one Turkish population from the Pontic Mountains ("Uzungöl-Trabzon", UzT) in June 2005 (Figure 1). Field trips within three regions in the eastern (Kakheti, Lg), central (Samtskhe-Javakhéti, Bk) and south-western parts (Adjara, Bt) of Georgia were conducted during summer 2008 and allowed collection of mature capsules (~50) with seeds (~500) from sixteen native populations (Figure 1 & Appendix 1). Moreover, during the field trips in the northern part of Georgia (Kazbegi, Kz) flowers (~2000) from seven native populations were collected (Appendix 1).

4.2.2 Sampling and crossing experiments

At least ten "putative" individuals representing the largest repartition of *V. filiformis* within each locality (see pop. area in Table 1) and without connection between them were collected in plastic bags with wet paper tissue in order to keep them alive during the collecting trip.

Afterwards, these plants were transplanted to a greenhouse of the botanical garden of the Johannes Gutenberg-University, Mainz, Germany. Each population was covered by tissue or plastic lids to prevent insect- or wind-pollination and each "putative" individual (10-12 per population) was potted and labeled separately. Fertilizers were used at the start of the experiment to improve the success of the transplantation. Flowers are slightly protogynous and the virginity of the stigma was checked by binoculars before crossing and generally, one anther with white mature pollen was used only one time to fertilize a single style using sterile forceps in order to deposit enough pollen per style and prevent other crossings. Labeled threads with a code were attached to the pedicels after crossing in order to identify the flowers later. The code, date of crossing, the father individual and its population together with the mother individual and its population were noted. Every week after a crossing, each labeled thread was checked in order to score the presence or absence of developing fruits. To confirm the absence of capsule production within introduced populations, eight specimens from the population "Pliezhausen" Pl were crossed together ($n_{flowers} = 41$). Introduced populations are adapted to their new climate and geographical conditions and have, therefore, asynchronous flowering periods making long-distance inter-population crossings difficult (Scalone, Kolf and Bull, unpubl. : Short Research Note "*Induction of flower production in* Veronica*"*). Thus, emphasis was laid on crossing neighboring populations. After that, populations from different crossing groups geographically distant were crossed with each other to check if they belong to the same crossing group or not (Figure 2).

4.2.3 DNA extraction and AFLP generation

A total of 108 DNA-samples considered as "putative" individuals were sampled from twenty introduced and three native populations (Table 1). DNA-samples do not correspond to the samples used for the crossing experiments although they come from the same German populations. Fresh leaves of the individuals were dried in silica gel and used to extract total genomic DNA by DNeasyTM plant minikit (Qiagen) following the manufacturer's instructions. DNA concentration was measured spectrophotometrically with a GeneQuant RNA/DNA calculator (Pharmacia), or estimated visually by ethidiumbromide staining on agarose gels. The AFLP procedure followed Vos *et al.* (1995) with modifications. Genomic DNA was digested with the two restriction endonucleases EcoRI and MseI and ligated to double-stranded EcoRI (5' – CTCGTAGACTGCGTACC – 3'; 3' – AATTGGTACGCAGTC - 5') and MseI (5' – GACGATGAGTCCTGAG – 3'; 3' – TACTCAGGACTCAT - 5') adapters in one step at 37°C for 3 hours. The reaction mix for circa 0.5 µg template DNA

contained 1.1µl T4 DNA ligase buffer (Genecraft), 1.1 µl 0.5 M NaCl, 0.55 µl bovin serum albumin (BSA, 1mg/ml, New England Biolabs, Beverly, Massachusetts), 1.0 µl 50 µM MseI-adapters (Metabion), 0.1µl MseI (10U/µl, New England Biolabs), 1.0 µl 5 µM EcoRI-adapters (Metabion), 0.25µl EcoRI (20U/µl, New England Biolabs), 0.002 µl T4 DNA ligase (10WU/µl, Genecraft) and 0.9 µl ddH2O. Ligated DNA fragments were diluted 10-fold. Preselective and selective amplifications were performed in a thermocycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, California) with PCR protocols following Vos *et al.* (1995) with modifications. The reaction mix for preselective amplification contained 2.5 µl 10× NebTaq PCR reaction buffer (New England Biolabs), 0.1 µl NebTaq PCR reaction mix (5U/µl, New England Biolabs), 0.25 µl 10 mM dNTPs (Applied Biosystems), 0.50 µl 5 µM preselective primers E01 (5' – GACTGCGTACCAATTCA – 3') and M02 (5' – GATGAGTCCTGAGTAAC – 3'), 6.65µl ddH2O and 2 µl diluted product of the restriction-ligation reaction. The PCR product was diluted 10-fold. The reaction mix for the selective amplification contained 1.667 µl 10× NebTaq PCR reaction buffer (New England Biolabs), 0.055 µl NebTaq PCR reaction mix (5U/µl, New England Biolabs), 0.167 µl 20 mM dNTPs (Applied Biosystems), 0.833 µl 50 mM MgCl2, 0.28 µl 5µM MseI-primer (Metabion), 0.20 µl 1µM EcoRI-primer (Metabion), 7.92 µl ddH2O and 5 µl diluted product of the preselective amplification. The six primer combinations for the selective PCR were E38 - Hex (5' Hex - GACTGCGTACCAATTCACT - 3') combined with M56 (5' - GATGAGTCCTGAGTAACGC - 3'); E38 - 6 - Fam / M50 (5' - GATGAGTCCTGAGTAACAT - 3') and E37 - Ned (5' Ned - GACTGCGTACCAATTCACG - 3') / M54 (5' - GATGAGTCCTGAGTAACCT - 3'); E38 - Hex combined with M62 (5' - GATGAGTCCTGAGTAACTT - 3'); E38 - 6 - Fam / M53 (5' - GATGAGTCCTGAGTAACCG - 3') and E37 - Ned / M51 (5' - GATGAGTCCTGAGTAACCA - 3'). 2.5 µl of 6-Fam - 3.75 µl of Hex and 3.75 µl of Ned labeled products of each sample were combined and 2 µl of this multiplex product was run with 7.75 µl HiDi formamide (Applied Biosystems) and 0.25 µl internal size standard GeneScan ROX (Applied Biosystems) on an ABI 3130x automated capillary sequencer. Raw AFLP data were collected and aligned with the internal size standard using ABI Prism GeneScan analysis software 3.7 (Applied Biosystems). Peaks (i.e. fragments) were scored manually as present (1) or absent (0) in a readable region of bands from 75 to 500 bp in length with GeneMarker version 1.5 (GeneMarker, SoftGenetics, LLC). Each peak superior to an intensity of signal of 1000 was selected and checked for each sample.

4.2.4 AFLP data analyses

At first, the mismatch error rate based on nine (8.3%) supplementary re-extracted samples (or replicates) belonging to one native and six introduced populations was calculated to evaluate the quality of our analyses and determine the clonality threshold as the number of genotype mismatches divided by the number of replicate pairs and the number of loci (Bonin *et al.,* 2004; Paun *et al.,* 2008; Table 1). The presence / absence matrix generated with the six primer combinations was imported in FAMD v. 1.1 (Schluter & Harris, 2006) to assess the genetic diversity between areas, regions, populations, crossing or genetic groups. The genetic diversity is represented, within these different levels of analysis, by the mean of AFLP fragments present per individual, by the number of polymorphic fragment (Frag. polym.) and its percentage (% Frag. polym.), by the number of fixed fragment (Frag. fix.), by the number of private fragment (Frag. priv.), by the number of private and fixed fragment and finally Nei´s gene diversity (Hj; Table 2). Analyses of molecular variance (AMOVA) were conducted using the same software and are represented in Table 3. To estimate if differences exist between samples (area, population, crossing and genetic groups) within the genetic data, the non-parametrical Wilcoxon-Mann-Whitney-test (W / U) for two groups or the Kruskall-Wallis-test (H) for more than two groups were conducted using SPSS v 15.0 software (SPSS Inc., Chicago, IL, USA). Genetic structure of populations was analyzed using the software STRUCTURE (vers. 2.3; Pritchard *et al.,* 2000). First, most of the parameters were set up as recommended in the user´s manual of STRUCTURE 2.0 (Pritchard *et al.,* 2000) and then, the option of "correlated allele frequencies" between populations was tested as recommended by Falush *et al.* (2003) in case of subtle population structure. From a pilot study, we found that a burn-in period and MCMC (Markov chain Monte-Carlo) of 10.000 and 50.000 iterations respectively, were sufficient. A series of 10 runs was conducted for each value of K populations between 2 to 23 (maximal number of localities) and the maximal value of Ln (Prob) obtained for each K was used to represent the best population structure acquired with the no-admixture and admixture models (Appendix 2). The no-admixture model is represented in Figure 3 and has been chosen since the amount of admixture was found to be negligible. A Principle Coordinates Analysis (PCoA) was performed using Nei & Li's coefficient (Nei & Li, 1979) and the coordinates of each individual were extracted (Figure 4_A & 4_B) to test statistically their distributions by the same Wilcoxon-Mann-Whitney and Kruskall-Wallis -tests using SPSS v. 15.0 (SPSS Inc.).

4.2.5 Flower characters

In the course of our crossing experiment, normal flowers (Figure 5_A & 5_B) and flowers with different types of mutations in the androecium (Figure 5_{C1-5}) were observed in the greenhouse and in the field. These different types of androecium-mutant flowers were searched and counted in ten introduced and seven native populations (Figure 3 and Appendix 1) in the field or greenhouse. To check if these flower mutations are specific to *V. filiformis* (*V.* subgenus *Pocilla*), these particular mutant flowers were counted in populations of two other *Veronica* species: the closely related *V. persica* (invasive in Europe; self-compatible; *V.* subgenus *Pocilla*) and *V. chamaedrys* (native in Europe; self-incompatible; *V.* subgenus *Chamaedrys*) growing both spontaneously in the same environment as another introduced *V. filiformis* population (arboretum of the botanical garden of Mainz; Mz). In our transplantation experiment, flower investment was estimated as the number of flowers produced per flowering specimen per population.

4.2.6 Pollen and ovule characters (P-O ratio)

The protocol for pollen-ovule ratio determination followed the one described by *Scalone et al.* (*subm.*). To estimate the investment in gametophytes, at least eight buds per population in the five native and eight introduced populations of *V. filiformis* were sampled for a total of 135 mature buds (Table 4). One-sample Kolmogorov-Smirnov test was conducted on the pollen and ovule production using SPSS (SPSS Inc.): pollen number follows a normal distribution after log-transformation (p-value < 0.05) whereas ovule number did not (p-value > 0.05). Thus, the non-parametrical Kruskal-Wallis-test (H) was used in SPSS (SPSS Inc.) to detect differences in ovule production between areas ("Native" Nat. / "Introduced" Intr.), regions ("Greater Caucasus" GC, "Lesser Caucasus" LC, "Baden-Württemberg" BW and "Bavaria" BV), population, crossing and genetic groups (Table 4 and Appendix 4_B) whereas a parametrical one-way ANOVA-test (F) was required for the analysis of pollen number (Table 4 and Appendix 4_A). To examine the relationships between pollen and ovule productions, the frequencies of mutant flowers, the flower investment, the genetic data (Hj and the percentages of polymorphic, private and fixed AFLP-fragments) and between the genetic and geographic distances within introduced populations, Spearman correlations were investigated using SPSS (SPSS Inc.) (Appendix 5 & 6_{A-E2}).

4.2.7 Seed characters

Seed number and size

Day by day after a compatible crossing, maturity of capsules was controlled until opening of the capsule. The seeds were collected in paper bags and stored in a dry place. The number of seeds per capsule was noted and the length and width of each seed were measured under a graduated microscope (Table 5 and Appendix 7). To observe differences in the sexual reproductive success the number and size of seeds from crossing experiments with introduced populations of Germany were compared statistically to the number and size of seeds collected from populations of three native regions in Georgia by the non-parametrical Kruskall-Wallis-test (H) using SPSS (Figure 6_A, Table 5, Appendix 4_B & 8_A & 8_B).

Seed viability

Seed viability was tested on seeds from crossings conducted between populations of the triangle "Tl-Pl-Re". These populations belong to different crossing groups (red, blue and green crossing groups; Figure 1) and different genetic clusters (Green, Blue and Pink genetic clusters; Figure 2). These "Tl-Pl-Re" groups were used to observe maternal and paternal effects on seed size, viability and embryo size within the introduced range (Appendix 9_A & 9_B). At least twenty seeds were tested in each combination of maternal and paternal parent ♀ - ♂: Tl-Pl, Pl-Tl, Tl-Re, Re-Tl, Pl-Re and Re-Pl to have equal number of seeds from the same mother and father population. Fifty seeds from two parts of Caucasian mountains in Georgia [from three regions Adjara, Samtskhe-Javakhéti and Kakheti localized respectively in the south-western and central parts of the Lesser Caucasus and in the eastern Georgian part of the Greater Caucasus] were used to compare seed viability between introduced and native ranges. The seed from the native area were also used to observe any significant differences in seed size, embryo size and seed viability between the native regions investigated (Appendix 1). A total of 150 seeds from the introduced area were compared with 150 seeds from the native area by a tetrazolium staining (ISTA 1985; Hampton & Tekrony, 1995; Leist & Krämer, 2003). Seeds were sliced longitudinally trough the midsection of the distal half, then placed in petri dishes with 2.5 ml of 1% solution by mass of 2,3,5 triphenyl tetrazolium chloride. After a 24h staining time in the germination room, seeds that displayed a completely red-stained embryo and endosperm were classified as viable (ISTA, 1985; Hampton & Tekrony, 1995; Leist & Krämer, 2003) (Appendix 10_A). The red color of the tissue indicates that the cells were respiring and living. Other distribution of color were considered as unviable ("abnormal seeds" corresponding to seeds having a red endosperm with a red and white embryo inside while "dead seeds" correspond to seeds with one or both organs white; Appendix 10_B & 10_{C1-3}). Seed viability was calculated as the number of viable seeds in the total of tested seeds for

each area (introduced / native) or each mother ♀ / father ♂ group ("Tübingen Lustnau" Tl / "Pliezhausen" Pl / "Reutlingen" Re) and statistically tested by the non-parametrical Wilcoxon-Mann-Whitney-test with the software SPSS (Figure 6_B and Appendix 11). During these seed dissections the length of the embryos of the tested seeds were measured under a graduated binocular (Table 6) and analyzed as for seed size (Appendix 4_B & 8_A & 8_B & 9_B).

Seed germination

A seed germination test (Baskin & Baskin, 1983; Nagel *et al.*, 2009) was conducted and adapted to our *V. filiformis* species. A set of Georgian seeds of *V. persica* were used to confirm the validity of this test after our modification (data not shown). 150 seeds of the native area from two of the three previously cited Georgian regions (Samtskhe-Javakhéti and Kakheti with three repeats of 25 seeds each) and 150 seeds of the introduced area from a random sample of introduced and crossed-compatible populations from inside and outside the transect (Baden-Württemberg and Bavaria) were investigated. Seeds were stored at 5°C for two weeks before being placed in wet Petri dishes (with fungicide added). The number of germinated and non germinated seeds was counted after a temperature treatment of 30°C - 8 hours / 15°C - 16 hours during a period of 28 days. The results were compared statistically by the non-parametrical Wilcoxon-Mann-Whitney-test using SPSS (Figure 6_C).

4.3 RESULTS

4.3.1 Crossing experiments

A total of 436 flowers were crossed in our intra- and inter-population crossing experiments.

Forty-one flowers from eight specimens of the population "Pliezhausen" Pl did not produce any capsule after intra-population crossings.

Based on the information provided by the inter-population crossing experiments, we designated crossing groups defined by sterility after intra-crossing group pollinations (dashed lines; Figure 2) and by seed production after inter-crossing group pollinations (solid line; Figure 2; 73.73% successful crossings). By this method, we could group 19 populations in five different crossing groups represented by different colors in Figure 2.

Thirteen populations from Baden-Württemberg to Bavaria belong to the same crossing group (green colour, Figure 2; "Kirchentellinsfurt" Kt, "Betzingen" Bz, "Reutlingen" Re, "Hohenwittlingen" Hw, "Blaubeuren" Bl, "Ulm" U, "Wiblingen" Wi, "Illerzell" Il, "Günzburg" Gu, "Burgau" Bg, "Ziemetshausen" Zi, "Westheim" We and "Augsburg" A). The absence of capsule production within these thirteen populations was observed after the

pollination of 143 flowers from 59 specimens with the pollen of 104 flowers from 43 specimens and corresponds to the 18 single and double dashed lines connecting all the "green" populations together in Figure 2. Moreover, seeds have been produced after the pollination of 63 flowers from 11 "green" populations with pollen from 18 specimens coming from other crossing groups and after the successful pollination of 31 plants from other crossing groups with pollen from 89 flowers of 11 "green" populations. These successful crossings, in which the "green" populations can be the receptor-plant (♀) or the donor-plant (♂), correspond to the 32 solid single and double lines connecting the "green" populations to other "no-green" populations in Figure 2.

Three populations from the most western part of our transet (Tp "Tübingen private garden", Tb "Tübingen old botanical garden" and Pl "Pliezhausen") form a second crossing group (blue color, Figure 2). This trio is based on the absence of seed production after the crossings of nine flowers, corresponding to the two dashed lines (one single and one double) connecting these three populations (Figure 2). This grouping is reinforced by the production of capsules after the crossings of 59 flowers of 16 specimens from this group with 20 plants from twelve populations belonging to four other crossing groups and after the successful pollinations of 51 flowers of 35 plants from other crossing groups by 13 specimens of these three populations (reference to the 23 solid single and double lines between the "blue" and the "no-blue" populations; Figure 2).

The other three populations form crossing groups of their own: "Tubingen lustnau" Tl (red, Figure 2), "Münsingen" Mü (pink, Figure 2) and "Mehrstetten" Mt (violet, Figure 2) since they produced fruits after all investigated inter-population crossings (Tl = 84 flowers from ten specimens used in crossings with thirteen different populations belonging to four other crossing groups; Mu = 48 fl., 11 pop.; Mt = 28 fl., eight pop.; Figure 2).

The population of "Großaitingen" Gß could not be grouped due to the lack of flowers in the field and in the greenhouse.

4.3.2 AFLP results

Population structure

The no-admixture analysis of STRUCTURE with correlated allele frequencies between populations obtained maximum likelihood scores for K = 6 to K = 10 (blue line; Appendix 2) whereas the admixture analysis with correlated allele frequencies inferred an optimal value of K = 9 (red line; Appendix 2). The minimal and optimal hypothesis (K = 6) found individuals of eight introduced populations belonging to just one genetic cluster ("Red" cluster: Bl / Il / Zi

; “Green” cluster: Tl / Hw ; “Blue” cluster: Tb / Pl ; “Pink” cluster: Re), individuals from eleven populations to belong to two different genetic clusters (“Red / Pink”: U / Wi / Gu / Bg ; “Red / Turquoise”: Kt / We / A ; “Red / Green”: Mu / Mt and “Pink / Blue”: Tp / Bz) and individuals from one population to belong to three genetic clusters (“Red / Pink / Blue”: Gß) (Figure 3). The three native populations belong to two different genetic clusters (“Turquoise” cluster: Kz9 and “Yellow” cluster: UzT / Bk10). The most widespread genetic clusters are the “Red” and “Pink” clusters found within thirteen and eight populations along a distance of 130 km from Kirchentellinsfurt to Augsburg and from Tübingen to Großaitingen (Figure 3). Rare AFLP-fragments (with allelic frequencies below to 0.15) represent 89% of the native area-specific fragments (40 on 45 AFLP-fragments) while they represent only 18% of the common fragments (22 on 121 AFLP-fragments). Moreover, fifteen of these 22 rare fragments common to both areas have a lower frequency within the introduced than within the native populations (68%; data not shown).

Clonality

Our AFLP primer combinations generate 294 unambiguous DNA-fragments from which 261 are polymorphic (88.78%). Four of them are fixed in *V. filiformis* and present in all DNA-samples (Table 2). The nine re-extracted individuals corresponding to our replicates gave an error rate of 4.65% (equal to 123 errors divided by nine pairs of replicate and 294 loci) according to Bonin *et al.* (2004). DNA-specimens which have a percentage of difference between their AFLP-fragments or a rate of pairwise individual comparisons below the error rate are considered as clones in this study. After two-by-two comparisons, 54 DNA-samples from 14 populations have been found to be clones (Table 1 & Figure 3). These 54 DNA-samples correspond to 10 different clones: nine clones are population-specific (c2 to c10; Figure 3) while one clone is present within eight different populations (c1; Tübingen private garden” Tp, “Betzingen” Bz, “Reutlingen” Re, “Ulm” U, “Wiblingen” Wi, “Gunzburg” Gu, “Burgau” Bg, “Großaitingen” Gs; Figure 3). This widely distributed clone represents almost all DNA-samples (N = 26 on 28) categorized by STRUCTURE as members of the “Pink” genetic cluster (Figure 3). Within our sampling, two populations are totally composed by a single clone (c1 in “Reutlingen” Re and c8 in “Ziemetshausen” Zi; Figure 3). In total, clonality was inferred for 58.06% of the DNA-samples from the introduced area (54 on 93 introduced specimens or DNA-samples) and 0.00% of the DNA-samples from the native region (0 on 15 native specimens).

Principle Coordinates Analysis

The PCoA based on standard similarity distances allow the visual differentiation of clusters in our data set in three axes (x, y, z) accounting for 28.80% of the molecular variability (12.92%, 9.34%, 6.54%) (Figure 4). Based on the PCoA coordinates, the native populations can be separated easily from the introduced ones (Mann-Whitney-U-test: U = 28.8, p-value < 0.0001; Figure 4_A). Within the introduced area, the groups of individuals based on the crossing experiment results (Figure 4_A) can be compared with the genetic clusters of the STRUCTURE analysis in Figure 4_B. Populations "Münsingen" Mü and "Mehrstetten" Mt belong to different crossing groups (pink and violet group, Figure 2) but cannot be distinguished from each other in the AFLP-analysis (p-value > 0.05; Figure 4_A). The same is true for the samples belonging to the blue and green crossing groups (p-value > 0.05; Figure 4_A). On the other side, the individuals of the "Hohenwittlingen" Hw population are significantly distant to the rest of the others individuals belonging to the green crossing group (U = 10.84, p-value < 0.001; Figure 4_A). Moreover, the samples of the "Großaitingen" Gß population belong to three different genetic clusters ("Pink", "Red" and "Blue"; Figure 4_B compared to Figure 4_A) but cannot be statistically differentiated ("Red"/"Pink": U = 7.35, p-value = 0.0067 but with "Red"/"Blue": U = 2.53, p-value = 0.1110; "Pink"/"Blue": U = 0.011, p-value = 0.917; Figure 4_B).

AMOVA and genetic diversity

Native populations have higher genetic diversity Hj (0.137) than introduced populations (0.0727; Wilcoxon-Mann-Whitney-test: U = 68, p-value = 0.018; Table 2) and twice the number of polymorphisms (28.23% versus 14.32%; p-value = 0.002; Table 2). In contrast, introduced populations possess more fixed fragments than native populations (51 versus 29; p-value = 0.002; Table 2).Within the introduced populations, four introduced populations do not have any private fragment ("Tübingen Lustnau", "Hohenwittlingen", "Ulm" and "Wiblingen") while "Augsburg" has the highest number of such fragments ($N_{priv.frag.} = 7$). The AMOVA-analysis found two third of the genetic variation within areas (67.35%; native and introduced) and only one third of the variation explained by differentiation between them (32.64%; Table 3). The six genetic clusters from the STRUCTURE-analysis explain approximately the same differentiation (within 70.86% / among 26.75% genetic clusters, data not shown). Within the introduced area a small difference in the repartition of the genetic variation is observed between our two different groupings: crossing groups or genetic clusters. There is more genetic variation within the five crossing groups (81.84%; Table 3) than among (18.16%) and also more within the five genetic clusters in the introduced populations (72.51%; Table 3) than among (27.49%). The genetic variation explained by within

population variation is higher in the native area (74.52%) than within the German populations (54.53%; Table 3).

4.3.3 Androecium-mutant flowers

The Mainz population of *V. filiformis* presents a higher percentage of mutant flowers (15.19%; N_{flower} = 953) than the Mainz populations of *V. chamaedrys* (1.85%; N_{flower} = 379) or *V. persica* (0.20%; N_{flower} = 507) from the arboretum part of the botanical garden. Among the ten investigated populations of *V. filiformis* in Germany (eight from the transect and one each in Bonn and Mainz; Figure 1 & 3), "Illerzell" Il is the population with the highest percentage of mutant flowers (47 in 103 observed flowers; 45.63%) with 36 flowers without filament (Figure 5_{C2}), nine with only one stamen (Figure 5_{C3}), one flower with two stamens of different sizes (Figure 5_{C1}) and the unique case observed of a flower with three stamens (Figure 5_{C5}). The population of "Mehrstetten" Mt has the lowest percentage of mutant flowers with 5.56% from a total of 54 counted flowers (Figure 3). Among the seven native populations investigated, only one has androecium-mutant flowers at low frenquency (1.48%; "Cross Pass" in the direction of Kobi Kz8; N_{flower} = 202). Variations in floral investment between the twenty introduced populations during the crossing experiment in 2007 and 2008 are represented in Appendix 3.

4.3.4 Pollen and ovule production

The native populations produce more pollen than the introduced population (one-way ANOVA: F = 29.489; p-value < 0.001; Table 4 and Appendix 4_A) but have significantly fewer ovules (Kruskal-Wallis-test: H = 4.899; p-value = 0.027; Appendix 4_B). The single native population with androecium-mutant flowers (Kz8) has a lower pollen production than the four other native populations (F = 6.263; p-value = 0.0015; Table 4 and Appendix 4_A) but no significant difference in ovule production (H = 5.730; p-value = 0.220; Table 4 and Appendix 4_B). Significant differences are observed in pollen production as a function of population (F = 8.252; p-value < 0.001) or genetic cluster (F = 3.519; p-value = 0.018; Table 4 and Appendix 4_A) in the introduced region but not as a function of crossing group (F = 2.981; p-value = 0.066; Table 4 and Appendix 4_A). Ovule number did not vary significantly between regions or populations in the native area but did so in the introduced range (Appendix 4_B).

4.3.5 Seed production

Capsules produced in the greenhouse after hand-pollination contained more seeds than capsules collected in the field after open-pollination (Kruskal-Wallis-test: H = 5.064; p-value

= 0.024; Table 5 & Appendix 4_B). Variations in seed number per capsule are found between native and introduced area and within this latter one between populations, crossing and genetic (Table 5 and Appendix 4_B). Seeds of the introduced area are smaller than seeds of the native area (Kruskal-Wallis-test: H = 88.053; p-value < 0.001) and these results are also observed for embryo size (H = 4.472; p-value = 0.034; Figure 6_A, Table 6, Appendix 4_B). Significant differences were found between Georgian populations for seed size (H = 169.202; p-value < 0.001; Appendix 4_B & 8_B), but not for embryo size (Appendix 4_B & 8_B). Between the two sets (native / introduced) of seeds, a significant difference was found in the number of dead seeds between the two areas (H = 1.84; p-value = 0.033; Figure 6_B) when the unviable seeds are separated in abnormal and dead seeds (Appendix 10_B & 10_{C1-3}). The abnormal seeds are likely viable and only stained insufficiently. Within the "Tl-Pl-Re" triangle of the introduced area seeds produced by the widespread "green" crossing and "Pink" genetic cluster (Figure 3) have a significant lower viability (22% of viable seeds / 78% of unviable seeds, green pop., Appendix 11) than the seeds produced by the two others crossing or genetic groups ("red" crossing and "Green" genetic groups: 44% viable / 56% unviable, t = -30.98; P-value = 0.001 and "blue" crossing and "Blue" genetic group : 36% viable / 64% unviable, U = -3.24; p-value = 0.042, Appendix 11) irrespective of the pollen donor (see below). A maternal effect is also found for seed and embryo sizes between populations belonging to the "blue" crossing or "Blue" genetic cluster producing significantly bigger seeds with bigger embryos than the ones from the others crossing or genetic groups (Appendix 9_A & 9_B). No paternal effect was found by comparing the results of viability, seed and embryo sizes between the same combinations ♀ - ♂: Tl-Pl / Pl-Tl; Tl-Re / Re-Tl and Pl-Re / Re-Pl (Appendix 9_A for seed and Appendix 9_B for embryo size; data not shown for seed viability). Moreover, the seeds from the native area have germinated more frequently than the ones from the introduced area during the three repeats (90% / 58%, 88% / 58%, 90% / 60%) and this difference of germination rate is statistically significant (H = 3.97; P-value = 0.046; Figure 6_C).

4.3.6 Correlations between data

Within the introduced populations a strong positive correlation exists between their genetic distances and their geographic distances (r = 0.293; p-value < 0.000; Appendix 5).

Positive correlations are observed between pollen production and a) Nei´s gene diversity (Hj; r = 0.722; p-value = 0.019, data not shown), b) the number of polymorphic (r = 0.695; p-value = 0.019; Appendix 6_{C1}) and c) private AFLP-fragments per population (r = 0.612; p-value = 0.040; data not shown). Negative correlations exist between the percentage of androecium-

mutant flowers and a) pollen production per flower and per population (r = - 0.508; p-value = 0.046; Appendix 6_A) and with b) number of polymorphic (r = - 0.579; p-value = 0.040; Appendix 6_{E1}) and c) number of private AFLP-fragments per population (r = - 0.592; p-value = 0.036; data not shown). Moreover, other negative correlations were found between pollen production per flower and the number of fixed AFLP-fragment (r = - 0.793; p-value = 0.005; Appendix 6_{C2}) and the percentage of clones per population (r = - 0.744; p-value = 0.011; Appendix 6_{D1}). Conversely, ovule production is not correlated with floral (rate of mutant flower per pop. in Appendix 6_B) or with the population genetic data (data not shown). It must be mentioned that the genetic diversity Hj is not correlated with the number of DNA-samples or specimens investigated per population (r = - 0.067; p-value = 0.380) but is significantly and positively correlated to the proportion of non-clonal specimens per population ($N_{\text{no clone}} / N_{\text{DNA sample}}$; r = 0.765; p-value < 0.001; Table 1) and negatively to the proportion of clones per population (r = - 0.800; p-value < 0.001).

4.4 DISCUSSION

4.4.1 An invasion without seed: Is V. filiformis sterile in Europe?

Since the first record of *Veronica filiformis* found around the roots of Georgian vine shoots imported after an epidemic of phylloxera in the neighborhoods of Marseille (Aubagne, southern France, 1893; Lehmann, 1942), European populations of this species have been considered sterile by botanists because no seed set has been observed in the introduced area (Lehmann, 1942; Thaler, 1953; Harris & Lovell, 1980[A]). Our intra-population crossing experiments within a German population (Pliezhausen) confirm previous unsuccessful and incompatible intra-population crossings conducted by Thaler (1953). However, the ability to set seed successfully has been demonstrated by our inter-population crossing experiments in the greenhouse. Therefore, the reproductive system of *Veronica filiformis* is still functional in Europe and the species is not sterile in its introduced area (Figure 2). Consequently, the widely accepted assumption that the invasion process of a self-incompatible species necessitates either the breakdown of the SI system or the disruption of the sexual reproduction as already observed in *Oxalis* sect. *Corniculatae* (Ornduff, 1972), *Eichhornia paniculata* (Barrett *et al.*, 1989), *Hypochaeris* sect. *Hypochaeris* (Ortiz *et al.*, 2006), *Centaurea solstitialis* (Sun & Ritland, 1998), *Senecio squalidus* (Hiscock, 2000) or *Aster furcatus* (Reinartz & Les, 1994) does not apply to *Veronica filiformis*.

4.4.2 Colonization by vegetative means: Is clonal dispersal in V. filiformis possible?

Several traits and circumstances come together to allow the colonization of Europe in the absence of seed set in *V. filiformis*. First, invasion without seed production has been possible because the species produces easily abundant adventitious roots on each node of the stem, which facilitates vegetative reproduction by fragmentation via cutting, mowing, raking or grazing. Indeed, only three days after cuttings almost all nodes of each new fragment of this creeping species produce adventitious roots (90%; Harris & Lovell, 1980[A] and personal observations done during the transplantation experiments) and facilitate the survival of the new "individuals". This stoloniferous habit is common in the genus (Harris & Lovell, 1980[B]) and in other self-incompatible weeds such as *Ranunculus repens* (Sarukhán, 1974) or *Trifolium repens* (Chapman, 1983). Second, *V. filiformis* can spread over more than 40 cm² per year and produces dozens of new fragments originating from one single individual with almost no mortality under standard conditions in the introduced area (Harris & Lovell, 1980[C] and personal observations). Its optimal growth is achieved under the following propitious environmental conditions: low summer temperatures with high rainfall (Harris & Lovell, 1980[C]) in ecosystems such as pasture lands, lawns and turfs (Salisbury, 1961; Müller, 1990; Perring, 1996). Similar environmental conditions are shared by the common turf community including *Cardamine pratensis, Holcus lanatus, Rumex anthora, Rhytidiadelphus squarrosus, Salvia pratensis, Symphytum officinalis, Taraxacum officinalis* and *Trifolium repens* (Lehmann, 1942; Thaler, 1953; personal observations). In these human-made habitats *V. filiformis* can easily be dispersed by humans, animals, wind or water depending on the environment (Harris & Lovell, 1980[C]), spread over several kilometres a year (2.47 km/y.; Telfer *et al.,* 2002; Williamson *et al.,* 2005; Walker, 2007) and infest these habitats by covering up to 80-90% of the grassed area (Kaufmann, 1979).

4.4.3 Are there clones of V. filiformis in Europe?

These two characteristics of *V. filiformis* provide an escape way for a self-incompatible species colonizing a new area. Wide spread of clones is revealed by AFLP-fingerprinting data in our study case. Our genetic analysis corroborates the common presence of clones including several monoclonal populations (Figure 3 & Table 1), whereas no clonal individuals have been found in populations of the native area. Similar results with clonal reproduction restricted to introduced populations have been found in other examples of invasive weeds such as *Rubus alceifolius* (Amsellem *et al.*, 2000, 2001) or *Fallopia japonica* (Hollingsworth & Bailey, 2000), although a larger number of native populations should be investigated genetically in order to confirm this conclusion. The presence of clones within our sampling is

also verified by the absence of a relationship between the number of genetically investigated individuals per population and the level of intra-population genetic diversity. However, such a correlation appears when clonal samples are deleted from the analysis (data not show). Moreover, the significant difference between the distributions of molecular variance among and within populations of two areas supports the hypothesis of a difference in population structure between the two areas. Indeed, the molecular variance is distributed equivalently among / within the native populations (between: 25.48% / within: 74.52%) and among / within the genetic clusters of the introduced area (between: 26.09% / within: 73.90%) compared with that among / within the introduced populations (between: 45.47% / within: 54.53%; Table 3). Although we have identified extensive clonal reproduction, intra-population genetic diversity within introduced population is not zero (Table 2) either i) due to the presence of several clones of multiple introductions leading to the question why sexual reproduction was not observed in these populations (Figure 3) or ii) due to the accumulation of somatic mutations within clones as suggested for the self-incompatible and asexual reproducting *Oxalis pes-caprae* (Rottenberg & Parker, 2004).

4.4.4 How many introductions of V. filiformis?

Our data indicates that *V. filiformis* spreads most likely by vegetative means in its introduced area. Indeed, AFLP-fingerprint data suggest according to the assignment test that two genetic clusters ("Red" cluster, thirteen populations between "Kirchentellinsfurt" and "Augsburg" / "Pink" cluster, eight populations between "Tübingen private garden" and "Großaitingen") have been dispersed over hundred kilometres (130 km) across the study area (Figure 3). These genetic clusters likely correspond to separate introductions, although this is likely an underestimate for the number of separate introductions since introductions of closely related plants cannot be distinguished (see below). The large zone covered by individuals belonging to the same genetic cluster in this non-native environment is accompanied by low frequency or absence of private fragments (Table 2). The complete absence of private fragments in the populations "Tübingen Lustnau", "Hohenwittlingen", "Ulm" and "Wiblingen" coincides with observations by Lehmann (1942) on site of first introductions of *V. filiformis* in Baden-Württemberg and Bavaria (Figure 1 and Table 1). If these populations are indeed the source of further introductions, all fragments initially present in these populations would be found in its satellite populations and the source populations would not contain any private fragment. These successive introductions during the colonization of southern Germany must have produced a succession of founder effects or bottlenecks as observed in *Fallopia sp.*

(Hollingsworth & Bailey, 2000) or *Aster furcatus* (Reinartz & Les, 1994). However, we were not able to distinguish the successive bottlenecks from the initial bottleneck, marked by fewer polymorphisms, lower intrapopulation genetic diversities (Table 2) and by the reduction of the frequency of rare fragments ($f < 0.15$; data not shown) in introduced populations compared to the native ones. The absence of significant discrimination between specimens belonging to the "Red", "Blue" and "Pink" genetic clusters in the PCoA analysis confirms the high relatedness between them and suggests that these genotypes likely came from several individuals of a single region, possibly introduced by a single plant trader (Figure 6_A & 6_B). In the light of these results, it is surprising that little congruence can be observed between the crossing groups and the genetic clusters. Individuals belonging to the "Pink" and "Red" genetic clusters largely correspond to the "green" crossing group, while the individuals of the "Blue" genetic cluster correspond to the "blue" crossing group (Figure 3). However, several incongruencies exist between crossing and genetic data: i) the populations "Tp" and "Bz" possess the same genetic composition ("Blue" and "Pink" clusters) but have different S-alleles ("blue" and "green" crossing groups; Figure 3); ii) the populations "Mu" and "Mt" again share the same genetic composition ("Red" and "Green" clusters) but have different S-alleles ("pink" and "violet" crossing groups; Figure 3) and none is the "green" one; iii) the population "Hw" is incompatible to other "green" populations although it is alone with the "Tl" population in the "Green" genetic clusters (Figure 3). To explain these incongruencies, we need to assume that different S-alleles originating from the same introduction became fixed in different populations (case i, ii) or the same S-alleles were introduced from different introductions (case iii). For populations "Mü", "Mt" and "Tl" an additional explanation would be post-introduction evolution at the S-locus leading to unique novel S-identities in these populations. Alternatively, some post-introduction sexual reproduction could have allowed genetic exchange, especially at the S-loceus, but the small population size would have led to a gradual fixation of two S-alleles in a particular population (S-Allee effect; Busch & Schoen, 2008; Levin *et al.*, 2009). Thus, we infer between three and five distinct introductions within the introduced area. The correlation found between geographic and genetic distances is characteristic to multiple introductions (Appendix 5) as already observed by (Amsellem *et al.*, 2000; Genton *et al.*, 2005). Moreover, three introduced populations ("Kt", "We" and "A") 120 kilometres apart from each other and having the highest genetic diversity of the German regions are grouped together with the native population located in the Greater Caucasus ("Kazbegi-Kobi") in the same genetic cluster ("Turquoise" cluster; Figure 3 and Table 2). This crucial detail gives an indication on the north-eastern Georgian origin of these German

populations and rejects the two other native regions (north-eastern Turkey and central Georgia) as putative sources. However, detecting precisely the sources of each introduced populations would require an analysis with a denser sampling in the native distribution area.

4.4.6 An invasion without seed: Is V. filiformis pollinated in Europe?

A critical aspect of the establishment of a self-incompatible plant in a new environment is the availability of suitable pollinator. Although, this topic was not investigated experimentally in this study, previous observations of Thaler (1951) affirm that *V. filiformis* is pollinated mainly by two small widespread groups of insect: the little house fly, *Fannia canicularis* (Diptera) and sawflies, *Athalia spp*. (Hymenoptera), species present throughout the Middle East and Europe. Studies of flight behaviour conducted by Zeil (1986) and Amano *et al.* (1999) demonstrated that these two species are territorial. Flies patrol well-defined areas (50x50 cm horizontal, 25 cm vertical) above landmarks (e.g., spot of blue *V. filiformis* flowers) chasing off any possible competitor. Thus, long distance pollinator movement between populations is unlikely. Even in the situation with three geographically close populations ("Tl-Pl-Re", minimum distance four kilometres) belonging to different crossing groups ("red"-"blue"-"green" groups, Figure 2), different genotype clusters ("Green"-"Blue"-"Pink" clusters, Figure 3) and demonstrated to produce seeds in the inter-population crossing experiments, no seed production was found in the field. Moreover, the majority of introduced populations are located in public or private gardens, turfs or parks which are mowed during the flowering period in the introduced area (Walker, 2007). Thus, it is likely that even rarely produced fruits are cut off before the end of seed maturation, which necessitates more than one month after successful pollinations (personal observations).

4.4.7 Consequences of an absence of sexual reproduction: Müller´s ratchet in action?

Since sexual reproduction does not occur in the introduced area anymore, selection pressure on primary sexual characters of the flower has ceased compared to the native area. Thus, mutations in the reproductive parts fail to have a fitness effect and are free to accumulate. This accumulation of deleterious mutations in organism without recombination has been termed Müller´s ratchet (Müller, 1964; Felsentein, 1974) and has been shown to lead to an increased extinction risk in asexual organisms (Lynch *et al.,* 1995). Indeed, a frequent presence of mutant flowers within introduced populations can be observed (between 5% and 45% of flowers are affected within fifteen populations investigated; Figure 3) compared to their rare occurence in the native range (~1.5% within one on seven populations). Our

investigations indicate that these androecium-mutations also affect pollen production of the flower. First, introduced populations produce a highly significant lower number of pollen grains (30% less; Table 4 and Appendix 4_A). Second, the single native Georgian population possessing androecium-mutant flowers ("Cross Pass" Kz8) has a significant lower pollen production than the other native populations (40% less; Table 4 and Appendix 4_A). Third, a negative correlation was found between the percentage of androecium-mutant flowers counted per population and the pollen produced by these populations ($r = - 0.5076$). Since we assumed an origin of the German populations from the Greater Caucasus, where the single native population with androecium-mutant flowers (Kz8) was found, it is possible that the genetic basis for the andreocium mutations was inherited from the source area. However, another possibility is that this population itself has a history of extensive vegetative reproduction with relaxed selection on floral reproductive characters since a partial alteration of the environment has been observed on the site (personal observations). Moreover, the frequency of these androecium-mutations in a population of *V. filiformis* has been compared to two additional species of *Veronica* living in the same environment (arboretum of the botanical garden of Mainz): an introduced population of the self-compatible Caucasian *Veronica persica* and a natural population of the self-incompatible European *Veronica chamaedrys*, which can also reproduce clonally (Boutin & Harper, 1991; Dale & Causton, 1992). The comparison shows that androecium-mutant flowers can also be found in other species of *Veronica* but the high percentage of these mutants (~20% of flowers per population) is exclusive to the non-sexual reproducing *V. filiformis*. Identical mutations were also observed before by Lehmann in introduced populations of *V. persica* but not quantified due to their rarity confirming our results for this species (Lehmann, 1909).

Although the accumulation of mutations affects clearly the male traits (stamen, pollen) necessary for sexual reproduction in *V. filiformis* and although female traits involved in sexual reproduction should be affected similarly, seed production is still possible in Europe as demonstrated by successful crossings in the greenhouse. These successful crossings between introduced populations produced more seeds per capsule than those counted in capsules collected in the native habitat (Table 5 and Appendix 4_B) indicating i) pollen limitation in the native habitat or ii) differences in ovule number between different genotypes (Table 4 and Appendix 4_B). Significant differences are also observed in seed size between the native and the introduced areas (Figure 6_A). Again, smaller size of seeds produced by crossings compared to the size of seeds collected in the field could be induced by i) different genotypes introduced, ii) the fixed inbreeding depression that occurred during the first post-introduction

generations, or iii) the effect of deleterious mutations accumulated by Müller´s ratchet during the clonal reproduction after the cessation of sexual reproduction. Significant variations in seed size between native populations (Table 5 and Appendix 4_B) confirm that introduction of small seeded genotypes is a possibility. However, since differences in seed size parallel those observed in embryo size (Figure 6_A), number of dead seeds (Figure 6_B) and germination rate (Figure 6_C) between seeds of the two areas, the reason for these differences are more likely found in the genetic consequences of vegetative reproduction. Especially, the higher percentage of dead seeds and reduced germination rate in the introduced area is most likely an effect of the accumulation of deleterious mutations by Müller´s ratchet indicating that both sexual parts of the plant are affected by the accumulation of deleterious mutations.

Similar to our observation, a relation was recently detected between additional petals flowers, pollen viability of *Ranunculus repens* and the age of the meadows where this species propagates vegetatively (Warren, 2009). Based on numerous life history traits, *Ranunculus repens* is similar to our *Veronica filiformis* model. This relation between age of meadows, pollen viability and petal number suggests that these exceptional floral phenotypes and the increased pollen abortion are induced by the accumulation and finally fixation of deleterious mutations. This study has the advantage of our study that ages for the populations were available based on known ages of the meadows. For most of the populations in our study, the time of colonization is not known and thus the gradual accumulation of mutations suggested by Müller´s ratchet not directly inferable. However, some indirect evidence for the gradual accumulation is possible to infer by the analysis of correlations between the proportion of androecium-mutant flowers, pollen production and genetic diversity. Pollen quantity per flower seems to be affected negatively by the proportion of clones ($r = - 0.807$; Appendix 6_{D1}) or by the relatedness between individuals (number of fixed fragments per population; $r = - 0.746$; Appendix 6_{C2}) present within populations and positively by the intrapopulation genetic diversity (number of polymorphic fragments per population; $r = 0.695$; Appendix 6_{C1}). It must be underlined that high intrapopulational genetic diversity present in the introduced populations of *V. filiformis* seems to be essential to a high production of flower (normal and mutant) per individual (H_j; $r = 0.604$ & number of polymorphic fragment; $r = 0.462$; Appendix 6_{E2}) as already observed in the self-incompatible and clonal species, *Calystegia collina* (Wolf *et al*, 2000). The results taken together suggest that the more clones are present in an introduced population of *V. filiformis*, less overall flowers but more mutant will be produced by the individuals of this population and less pollen grains by these flowers. However, the underlying common cause for these observations are difficult to discern with

the most likely and not mutually excluding explanations being accumulation of deleterious mutations and fixations of the effects of inbreeding depression in the early generations after introduction when sexual reproduction may still have been possible.

To conclude, *Veronica filiformis* proved to be an excellent example to demonstrate the consequences of human introduction to a new environment. This obligate self-incompatible species which is native from the high mountains around the Black Sea colonized Europe and North America after introduction by gardeners or plant traders at the start of the last century, apparently exclusively without seed set. Nevertheless, watering, fertilizing and mowing of the European turfs facilitated the spread of clones of *V. filiformis* across more than 100 kilometers but without sexual reproduction even when compatible clones are less than four kilometres apart. However, this spread came at a prize, accumulation of deleterious mutations following Müller´s ratchet. This accumulation of mutations is detectable in various parts of the flower (androecium, pollen, ovule, seed, embryo) which lost in Europe its fundamental and evolutionary role for population maintenance.

4.5 ACKNOWLEDGEMENTS

We thank Prof. J.W. Kadereit for his guidance during this project, Dr. E. Westberg for the asssistance with the STRUCTURE analysis and M. Kever & S. Kempen from the Institut für Biologie und Umweltwissenschaften (IBU, Oldenburg, Germany) for help with pollen and ovule counts. We are grateful to Dr. U. Lohwasser and their assistants of the Leibniz Institute of Plant Genetics and Crop Research Science (Gatersleben, Germany) for support with the seed viability test by tetrazolium and for their precious recommendations for the seed germination test. We thank Dr. S. Shetekauri, Mr. T. Shetekauri and Mr. V. Kistauri for their assistance during the collection trip in Georgia too. This study was supported by the DFG (AL632 / 1-1) and the FWF (P18598).

4.6 REFERENCES

Amano T., Nishida R., Kuwahara Y., Fukami H. (1999) Pharmacophagous acquisition of clerodendrins by the turnip sawfly (*Athalia rosae ruficornis*) and their role in the mating behavior. *Chemoecology* **9:** 145-150.

Amsellem L., Noyer J., Le Bourgeois T., Hossaert-McKey M. (2000) Comparison of genetic diversity of the invasive weed *Rubus alceifolius* Poir. (Rosaceae) in its native range and in areas of introduction, using amplified fragment length polymorphism (AFLP) markers. *Molecular Ecology* **9:** 443-455.

Amsellem L., Noyer J., Hossaert-McKey M. (2001) Evidence for a switch in the reproductive biology of *Rubus alceifolius* (Rosaceae) towards apomixis, between its native range and its area of introduction. *American Journal of Botany* **88:** 2243-2251.

Baker H.G. (1955) Self-compatibility and establishment after "long-distance" dispersal. *Evolution* **9:** 347-349.

Baker H.G. (1967) Support for Baker's law-as a rule. *Evolution* **21:** 853-856.

Baker H.G. (1974) The evolution of weeds. *Annual Review of Ecology and Systematics* **5:** 1-24.

Bangerter E. B. and Kent D. H. (1957) *Veronica filiformis* Smith in the british isles. *Proceedings of the Botanical Society of the British Isles* **2:** 197-217.

Bangerter E. B. and Kent D. H. (1962) Further notes on *Veronica filiformis*. *Proceedings of the Botanical Society of the British Isles* **4:** 384-397.

Bangerter E. B. and Kent D. H. (1965) Additional notes on *Veronica filiformis*. *Proceedings of the Botanical Society of the British Isles* **6:** 113-118.

Barrett S., Morgan M., Husband B. (1989) The dissolution of a complex genetic polymorphism: The evolution of self-fertilization in tristylous *Eichhornia paniculata* (Pontederiaceae). *Evolution* **43:** 1398-1416.

Baskin J. and Baskin C. (1983) Germination ecology of *Veronica agrestis*. *Journal of Ecology* **71:** 57-68.

Blackburn T. M., Pyšek P., Bacher S., Carlton J. T., Duncan R. P., Jarošík V., Wilson J. R. U., Richardson D. M. (2011) A proposed unified framework for biological invasions. *Trends in Ecology and Evolution* **26:** 333–339.

Bleeker W. (2003) Hybridization and *Rorippa austriaca* (Brassicaceae) invasion in Germany. *Molecular Ecology* **12:** 1831-1841.

Bonin A., Bellemain E., P. Bronken Eidesen P., Pompanon F., Brochmann C., Taberlet P. (2004) How to track and assess genotyping errors in population genetics studies. *Molecular Ecology* **13:** 3261-3273.

Boutin C. and Harper J. (1991) A comparative study of the population dynamics of five species of *Veronica* in natural habitats. *Journal of Ecology* **79:** 199-221

Busch J. and Schoen D. (2008) The evolution of self-incompatibility when mates are limiting. *Trends in Plant Science* **13:** 128-136.

Byers D. and Meagher T. (1992) Mate availability in small populationsof plant species with homomorphic sporophytic self-incompatibility. *Heredity* **68:** 353-359.

Chapman D. (1983) Growth and demography of *Trifolium repens* stolons in grazed hill pastures. *Journal of Applied Ecology* **20:** 597-608.

Courchamp F., Berec L., Gascoigne J. (2008) Allee effects in ecology and conservation. Oxford University Press, New York. 255 pages.

Culley T. and Hardiman N. (2009) The role of intraspecific hybridization in the evolution of invasiveness: a case study of the ornamental pear tree *Pyrus calleryana*. *Biological Invasions* **11:** 1107-1119.

Dale M. and Causton D. (1992) The ecophysiology of *Veronica chamaedrys*, *V. montana* and *V. officinalis*. III. Effects of shading on the phenology of biomass allocations – a field experiment. *Journal of Ecology* **80:** 505-515.

Davis H., Taylor C., Civille J., Strong D. (2004) An Allee effect at the front of a plant invasion: *Spartina* in a Pacific estuary. *Journal of Ecology* **92:** 321-327.

Eckert C. (2002) The loss of sex in clonal plants. *Evolutionary Ecology* **15:** 501-520.

Falush D., Stephens M., Pritchard J. (2003) Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. *Genetics* **164:** 1567-1587.

Felsenstein J. (1974) The evolutionary advantage of the recombination. *Genetics* **78:** 737-754.

Gabrielsen T. and Brochmann C. (1998) Sex after all: high levels of diversity in the artic clonal plant *Saxifraga cernua* using RAPD markers. *Molecular Ecology* **7:** 1701-1708.

Genton B., Shykoff J., Giraud T. (2005) High genetic diversity in French invasive populations of common ragweed, *Ambrosia artemisiifolia*, as a result of multiple sources of introduction. *Molecular Ecology* **14:** 4275-4285.

Glémin S., Petit C., Maurice S., Mignot A. (2008) Consequences of low mate availability in the rare self-incompatible species *Brassica insularis*. *Conservation Biology* **22:** 216-221.

Grimsby J., Tsirelson D., Gammon M., Kesseli R. (2007) Genetic diversity and clonal versus sexual reproduction in *Fallopia spp.* (Polygonaceae). *American Journal of Botany* **94:** 957-964.

Hampton J. G. and Tekrony D. M. (1995) Handbook of Vigour Test Methods. International Seed Testing Association, Zürich.

Harris G. R. and Lovell P. H. (1980[A]) Localized spread of *Veronica filiformis*, *V. agrestis* and *V. persica*. *Journal of Applied Ecology* **17:** 815-826.

Harris G. R. and Lovell P. H. (1980[B]) Growth and reproductive strategy in *Veronica* spp. *Annals of Botany* **45:** 447-458.

Harris G. R. and Lovell P. H. (1980[C]) Adventitious root formation in *Veronica* spp. *Annals of Botany* **45:** 459-468.

Hiscock S. (2000) Genetic control of self-incompatibility in *Senecio squalidus* L. (Asteraceae): a successful colonizing species. *Heredity* **85:** 10-19.

Hollingsworth M. and Bailey J. (2000) Hybridisation and clonal diversity in some introduced *Fallopia* species. *Watsonia* **23:** 111-121.

Hollingsworth M. and Bailey J. (2008) Evidence for massive clonal growth in the invasive weed *Fallopia japonica* (Japanese Knotweed). *Botanical Journal of Linnean Society* **133:** 463-472.

ISTA (1985) International rules for seed testing. *Seed Sciences Technology* **13:** 299-513.

Kaufmann J. (1979) Control of creeping speedwell in turfgrass. Weeds Today (Automn 1979), 18-19.

Lamont B., Klinkhamer P., Witkowski (1993) Population fragmentation may reduce fertility to zero in Banksia goodie: a demonstration of the Allee effect. *Oecologia* **94:** 446-450

Lawrence M. (2000) Population genetics of the homomorphic self-incompatibility polymorphisms in flowering plants. *Annals of Botany* **85:** 221-226.

Lehmann E. (1909) Über Zwischenrassen in der *Veronica*-Gruppe *agrestis*. *Zeitschritft für indukt. Abstammungs- und Vererbungslehre* **2:** 145-208.

Lehmann E. (1942) Die Einbürgerung von *Veronica filiformis* Sm. In Westeuropa und ein Vergleich ihres Verhaltens mit dem der *V. tournefortii* Gm. *Gartenbauwissenschaften* **16:** 428-487.

Lehmann E. (1944) *Veronica filiformis* Sm., eine selbsterile Pflanze. *Jahrbücher für wissenschaftliche. Botanik* **91:** 395-403.

Lehmann E. and Schmitz-Lohner M. (1954) Entwicklung und Polyploidie in der *Veronica*-Gruppe *Agrestis. Zeitschrift für induktive Abstammungs- und Vererbungslehre* **86:** 1–34.

Leist N. and Krämer S. (2003) ISTA Working Sheets on Tetrazolium Testing, Volume I, 1st Edition - Agricultural, Vegetable and Horticultural Species.

Levin D., Kelley C., Sarkar S. (2009) Enhancement of Allee effects in plants due to self-incompatibility alleles. *Journal of Ecology* **97:** 518–527.

Li A. and Ge S. (2001) Genetic variation and clonal diversity of *Psammochloa villosa* (Poaceae) detected by ISSR markers. *Annals of Botany* **87:** 585-590.

Lynch M., Conery J., Bürger R. (1995) Mutational meltdowns in sexual populations. *Evolution* **49:** 1067-1080.

Müller H. (1964) The relation of recombination to mutational advance. *Mutation Research / Fundamental and Molecular Mechanisms of Mutagenesis* **1:** 2-9.

Müller N. (1990) Lawns in German cities. A phytosociological comparison. Urban ecology. SPB Academic Publishing bv, The Hague, The Netherlands: 209-222.

Nagel M., Vogel H., Landjeva S., Buck-Sorlin G., Lohwasser U., Scholz U., Börner A. (2009) Seed conservation in *ex situ* genebanks-genetic studies on longevity in barley. *Euphytica* **170:** 5-14.

Nei M. and Li W.-H. (1979) Mathematical model for studying genetic variation in terms of restriction endomucleases. *Proceedings of the National Academy of Sciences of the United States of America* **76:** 5269-5273.

Novak S. (2007) The role of evolution in the invasion process. *Proceedings of the National Academy of Sciences of the United States of America* **104:** 3671-3672.

Ornduff R. (1969) Reproductive biology in relation to systematics. *Taxon* **18:** 121–133

Ornduff R. (1972) The breakdown of trimorphic incompatibility in *Oxalis* section *Corniculatae. Evolution* **26:** 52-65.

Ortiz M., Talavera S., Garcia-Castano J., Tremetsberger K., Stuessy T., Balao F., Casimiro-Soriguer R. (2006) Self-incompatibility and floral parameters in *Hypochaeris* sect. *Hypochaeris* (Asteraceae). *American Journal of Botany* **93:** 234-244.

Paun, O., Schönswetter P., Winkler M., Tribsch A., Intrabiodiv Consortiun. (2008) Historical divergence vs. contemporary gene flow: evolutionary history of the calcicole *Ranunculus alpestris* group (Ranunculaceae) in the European Alps and the Carpathians. *Molecular Ecology* **19:** 4263-4275.

Perring F. (1996) The silent invasion. The Garden (June). 360-363.

Pimentel D., Lach L., Zuniga R., Morrison D. (2000). Environmental and economic costs associated with non-indigenous species in the United States. *BioScience* **50:** 53-65.

Poulin J., Weller S., Sakai A. (2005) Genetic diversity does not affect the invasiveness of fountain grass (*Pennisetum setaceum*) in Arizona, California and Hawaii. *Diversity and Distributions* **11:** 241-247.

Pritchard J., Stephens M., Donnelly P. (2000) Inference of population structure using multilocus genotype data. *Genetics* **155:** 945-959.

Rambuda T. and Johnson S. (2004) Breeding systems of invasive alien plants in South Africa: does Baker's rule apply? *Diversity and Distributions* **10:** 409-416.

Reinartz J. and Les D. (1994) Bottleneck-induced dissolution of self-incompatibility and breeding system consequences in *Aster furcatus* (Asteraceae). *American Journal of Botany* **81:** 446-455.

Rottenberg A. and Parker J. (2004) Asexual populations of the invasive weed *Oxalis pes-caprae* are genetically variable. *Proceedings of the Royal Society of London. B: Biological Sciences* **271:** S206-S208.

Salisbury E. (1961). Weeds & Aliens. New Naturalist Series, Collins, London.

Sarukhán J. (1974) Studies on plant demography: *Ranunculus repens* L., *R. bulbosus* L. and *R. acris* L.: II-Reproductive strategies and seed population dynamics. *Journal of Ecology* **62:** 151-177

Schlüter P. and Harris S. (2006) Analysis of multilocus fingerprinting data sets containing missing data. *Molecular Ecology Notes* **6:** 569-572.

Stebbins L. (1957) Self fertilization and population variability in the higher plants. *The American Naturalist* **91:** 337-354.

Sun M. and Ritland K. (1998) Mating system of yellow starthistle (*Centaurea solstitialis*), a successful colonizer in North America. *Heredity* **80:** 225-232.

Takebayashi N. and Morrell P. (2001) Is self-fertilization an evolutionary dead end? Revisiting an old hypothesis with genetic theories and a macroevolutionary approach. *American Journal of Botany* **88:** 1143-1150.

Telfer M., Preston C., Rothery P. (2002) A general method for measuring relative change in range size from biological atlas data. *Biological Conservation* **107:** 99-109.

Thaler I. (1951) Morphologisches über *Veronica filiformis* Smith und ihre Verwandten. *Phyton* **3:** 216-226.

Thaler I. (1953) Die Ausbreitung von *Veronica filiformis* Smith. *Phyton* **5:** 41-54.

Tiébré M.-S., Bizoux J.-P., Hardy O., Bailey J., Mahy G. (2007) Hybridization and morphogenetic variation in the invasive alien Fallopia (Polygonaceae) complex in Belgium. *American Journal of Botany* **94:** 1900-1910.

Vos P., Hogers R., Bleeker M., Reijans M., Van de Lee T., Hornes M., Friters A., Pot J., Paleman J., Kuiper M., Zabeau M. (1995) AFLP: a new technique for DNA fingerprint. *Nucleic Acids Research* **23:** 4407-4414.

Walker K. (2007) The last thirty five years: recent changes in the flora of the British Isles. *Watsonia* **26:** 291-302.

Warren J. (2009) Extra petals in the buttercup (*Ranunculus repens*) provide a quick method to estimate the age of meadows. *Annals of Botany* **104:** 785-788.

Williamson M., Pysek P., Jarosik V. and Prach K. (2005) On the rates and patterns of spread of alien plants in the Czech Republic, Britain and Ireland. *Ecoscience* **12:** 424-433.
Wolf A., Harrison S., Hamrick J. (2000) Influence of habitat patchiness on genetic diversity and spatial structure of a serpentine endemic plant. *Conservation Biology* **14:** 454-463
Wright J.T. and Davis A.R. (2006) Demographic feedback between clonal growth and fragmentation in an invasive seaweed. *Ecology* **87:** 1744-1754.
Zeil J. (1986) The territorial flight of male jouseflies (*Fannia canicularis L.*) *Behavioral Ecology and Sociobiology* **19:** 213-219.

4.7 CONTENTS

- **Figure legends**

Fig. 1. Distribution of *V. filiformis* in the native (green zone) and introduced (red zone) areas
The red points correspond to the introduced populations of *V. filiformis* mentioned in the literature of the introduction. The orange and green crosses correspond to the populations used during this study from the introduced and the native areas respectively.
Bk = Bakuriani (Samtskhe-Javakhéti region), Bo = Bonn (North Rhine-Westphalia region), Bt = Batumi (Adjara region), Kz = Kazbegi (Kazbegi region), Lg = Lagodekhi (Kakheti region), Mz = Mainz (Rhineland-Palatinate region), T-U-A = transect Tübingen-Ulm-Augsburg, (Baden-Württemberg and Bavaria regions), UzT = Uzungöl-Trabzon (Trabzon region).

Fig. 2. Crossing polygon indicating results from interpopulation crossings
The color of the circles corresponds to the crossing groups inferred by the crossing experiment. The name of populations corresponds to the code in Table 1. A double line indicates that the crossings were done in both directions (♀ pop. X with ♂ pop. Y as well as ♂ pop X with ♀pop. Y), whereas unique single line means that only one direction of the cross was conducted. The width of the lines is proportional to the number of flowers tested for this crossing. The dashed lines represent the incompatible crossings, whereas solid lines represent compatible crossings leading to the formation of seeds.

Fig. 3. Map of the transect
The color of the localities within the map corresponds to the crossing groups of populations determined by the crossing experiment (Figure 2). The colored bars outside the map refer to the genetic clusters inferred by STRUCTURE with no-admixture. The pie charts outside the

map indicate the proportion of normal (blue) and androecium-mutant (red) flowers per population. The names of populations follow those given in Table 1.

Fig. 4. Principle coordinate analysis of *V. filiformis* using standard similarity distances

A. Discrimination based on geographic region and crossing group
B. Discrimination based on genetic cluster

Colors for native populations correspond to the geographic distribution in Figure A and to genetic clusters identified during the STRUCTURE analyses of the AFLP experiment in part B. Colors for introduced populations correspond to crossing groups identified during the crossing experiments in part A and to genetic clusters identified in the STRUCTURE analyses of the AFLP experiment in part B. Black circles are individuals without crossing data from the Großaitingen population in part A and unclassified sample from the STRUCTURE analyses of the AFLP experiment (Mü) in part B. The dotted line corresponds to separate clusters highly supported (see Results for details).
Hw = Hohenwittlingen; Tl = Tübingen Lustnau; Mü = Münsingen; Mt = Mehrstetten.

Fig. 5. Morphology of normal and abnormal flowers in *V. filiformis*

A. Normal and radial flower with four petals, two equal stamens and one style.
B. Normal flower conserved in alcohol.
C. Androecium-mutant flowers observed in the introduced area (C_1. Flower with one long and one short filament, C_2. Flower without filament, C_3. Flower with one stamen, C_4. Flower without stamen, C_5. Flower with three stamens).

The scale corresponds to a length of 1 mm.

Fig. 6. Seed variations between native and introduced populations in *V. filiformis*

A. Seed and embryo size.
B. Seed viability test.
C. Seed germination test.

Significance level: * = $P \leq 0.05$, ** = $P \leq 0.01$ and *** = $P \leq 0.001$; grad. = graduations; 1 grad. = 0.12 mm.

- **Table legends**

Table 1. Information on the origin of samples used for the AFLP fingerprint analysis, mutant-flower investigation and pollen and ovule counts

The population in bold is the population which did not produce flowers during our crossing experiment. DNA-samples with one or several stars are repeats in the AFLP analysis used for error rate estimation. The number of stars corresponds to the number of re-extracted DNA samples in the respective population. Populations in italics are the populations used for pollen and ovule counts while the frequency of androecium-mutant flowers was estimated in underlined populations.

[1] = years obtained from literature (Lehmann, 1909; Thaler, 1939); [2] = years obtained from observations of local botanists (M. Thiv, Stuttgart, pers. comm.); N_{AFLP} = number of DNA-samples used for the AFLP experiment, 1st obs. = date of the first observations in the introduced area; Pop. area = population size estimation; BW = Baden-Württemberg; BV = Bavaria.

Table 2. Population genetic indices for the populations of *V. filiformis*

$n_{pop.}$ = number of population per area; Code = code of the population (see Table 1); $N_{samples}$ = number of DNA-samples per population; Frag. polym. = number of polymorphic AFLP-fragments; % Frag. polym. = percentage of polymorphic AFLP-fragments; Frag. fixed = number of fixed AFLP-fragments; Frag. priv. = number of private AFLP-fragments (number of private & fixed AFLP-fragments); S.D. = standard deviation; Var. = variance.

Table 3. Analyses of molecular variance (AMOVA) from AFLP fingerprints

Nat. = native area; Intr. = Introduced area; Anat. = Anatolian –Pontic mountains (Turkish population); G+L Cauc. = Greater and Lesser Caucasian mountains (Kazbegi and Bakuriani populations); g / b / r / p / v = green, blue, red, pink and violet crossing groups, respectively; P / T / B / Y / G / R = Pink, Turquoise, Blue, Yellow, Green and Red genetic clusters, respectively; N = number of groups; d.f. = degree of freedom; SS = sum of squares; V_{total} = variance total.

Table 4. Pollen and ovule productions in *V. filiformis*

$N_{flowers}$ = number of flowers dissected; Pollen = pollen number; Ovule = ovule number; S.D. = standard deviation; % S.D. = percentage of the standard deviation. The names of colors starting without capital letter correspond to the different crossing groups (Cros. group) while the names starting with capital letter correspond to the genetic clusters (Gen. group), as presented in Figure 2.

Table 5. Seed size and seed number per capsule in *V. filiformis*

$N_{caps.}$ = number of capsules collected in the native area or obtained during the crossing experiment; $N_{Seeds/Caps.}$. = number of seeds per capsule and per population; S.D. = standard deviation; % S.D. = percentage of the standard deviation; Total N_{seed} = total number of seeds measured per population. Seed length and width were measured under a graduated microscope and converted in mm.

Table 6. Embryo size in *V. filiformis*

Population = number of native populations or name of introduced populations studied; N_{embryo} = number of embryos measured; Embryo = embryo length; S.D. = standard deviation; % S.D. = percentage of the standard deviation.

Embryo length was measured after seed dissection necessary in the seed viability experiment. The information about population, crossing or genetic group define the three same clusters.

- **Appendix legends**

Appendix 1. Sampling of DNA, flower (mutant flower), bud, capsule and seed data from the native area

Appendix 2. Representation of the score of Ln (Prob) in function of the K-hypothesis obtained with admixture (blue) or no-admixture (red) models for the option of correlated allele frequencies

MixCorrel = no-admixture model with correlated allele frequencies; AdmixCorrel = admixture model with correlated allele frequencies.

Appendix 3. Floral investment of twenty introduced populations during the transplantation experiments in 2007 and 2008

Appendix 4. Statistical analyses of pollen, ovule and seed production, seed and embryo size

A) Statistical analyses of pollen number.
B) Statistical analyses of ovule and seed numbers, seed and embryo sizes.

A parametrical one-way ANOVA (F) was used to discriminate between differences in the pollen production after a log transformation while a non-parametrical Kruskal-Wallis-test (H) was used to discriminate between differences in ovule number, seed number per capsule, seed size, as well as embryo size between areas, regions, populations, crossing or genetic groups:

the significance levels are * = P ≤ 0.05, ** = P ≤ 0.01 and *** = P ≤ 0.001. Only the statistical analyses of differences observed in seed length are represented in Appendix 4_B (conclusions obtained with seed width are exactly identical; data not shown).

A non-parametrical Kruskal-Wallis-test (H) was used to discriminate between differences in seed number per capsule: the significance levels are * = P ≤ 0.05, ** = P ≤ 0.01 and *** = P ≤ 0.001.

Apppendix 5. Relationship between genetic and geographic distances within twenty introduced populations of *V. filiformis*

Spearman correlation between genetic and geographic distances gave:

r = 0.2925, N = 190, p < 0.0001***

N= number of couple of populations whose the genetic and geographic distances have been measured within German transect; Significance level: * = P ≤ 0.05, ** = P ≤ 0.01 and *** = P ≤ 0.001.

Appendix 6. Relationships between the percentage of androecium mutant-flower, pollen and ovule number, flower investment and genetic data

A. Relationship between the percentage of androecium mutant-flowers per population and pollen production

B. Relationship between the percentage of androecium mutant-flowers per population and ovule production

C_1. Relationship between pollen production and the number of polymorphic AFLP-fragment per population

C_2. Relationship between pollen production and the number of fixed AFLP-fragment per population

D_1. Relationship between the number of clones per population and pollen production

D_2. Relationship between the number of clones per population and seed number per capsule according to maternal population

E_1. Relationship between the number of polymorphic AFLP-fragment per population and the percentage of androecium mutant-flowers

E_2. Relationship between the number of polymorphic AFLP-fragment per population and the flower investment

Spearman correlations are indicated for each relationship as "R^2 =".

Appendix 7. Different types of seed produced during our crossing experiments from introduced populations (A, B and C) or collected in the native range (D)

Appendix 8. Variations of seed and embryo sizes in introduced (A) and native (B) populations

The measures were realized with one graduated microscope and are represented on Appendix 8_A and 8_B by graduation numbers (1 grad. = 0.12mm). The seed size corresponds to the length, although similar conclusions can be made with the data of seed width. In the introduced populations, the conclusions made from embryo and seed sizes are identical (A), while they are different in the native populations (B). Wilcoxon-Mann-Whitney-test compares the means of each observed characters for the three experiments: the means are significantly different when the P-value is ≤ 0.05 (*), with a P-value ≤ 0.01 (**), with a P-value ≤ 0.001 (***) or are not significantly different when the P-value is > 0.05 (ns)..

Appendix 9. Maternal and paternal effect in seed (A) and embryo size (B)

The population code correspond to the mother population (receptor plant) and the second code to the father population (donor plant) : ♀ x ♂. The colors correspond to the colors of the crossing groups (Tl = red, Pl = blue, Re = green). Wilcoxon-Mann-Whitney-test compares the means of each observed characters for the three experiments: the means are significantly different when the P-value is ≤ 0.05 (*), with a P-value ≤ 0.01 (**), with a P-value ≤ 0.001 (***) or are not significantly different when the P-value is > 0.05 (ns). 1 grad. = 0.12mm.

Appendix 10. Observations of endosperm and embryo in the seed viability test using tetrazolium

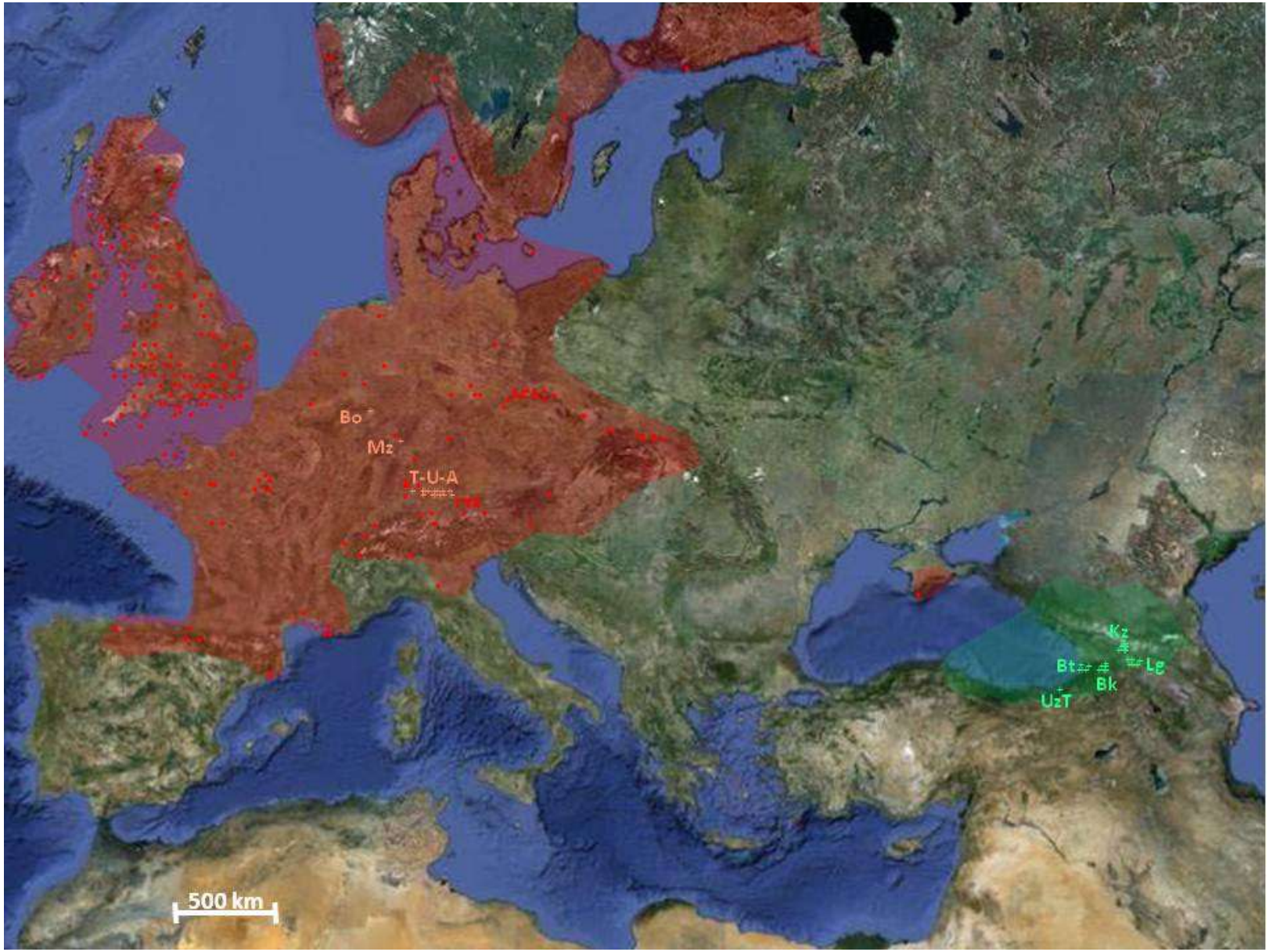
A. Viable seed (endosperm and embryo red).
B. Abnormal seed (red endosperm with red and white embryo).
C. Dead seeds (C_1. Red endosperm with white embryo; C_2. White endosperm with red embryo; C_3. Endosperm and embryo white).

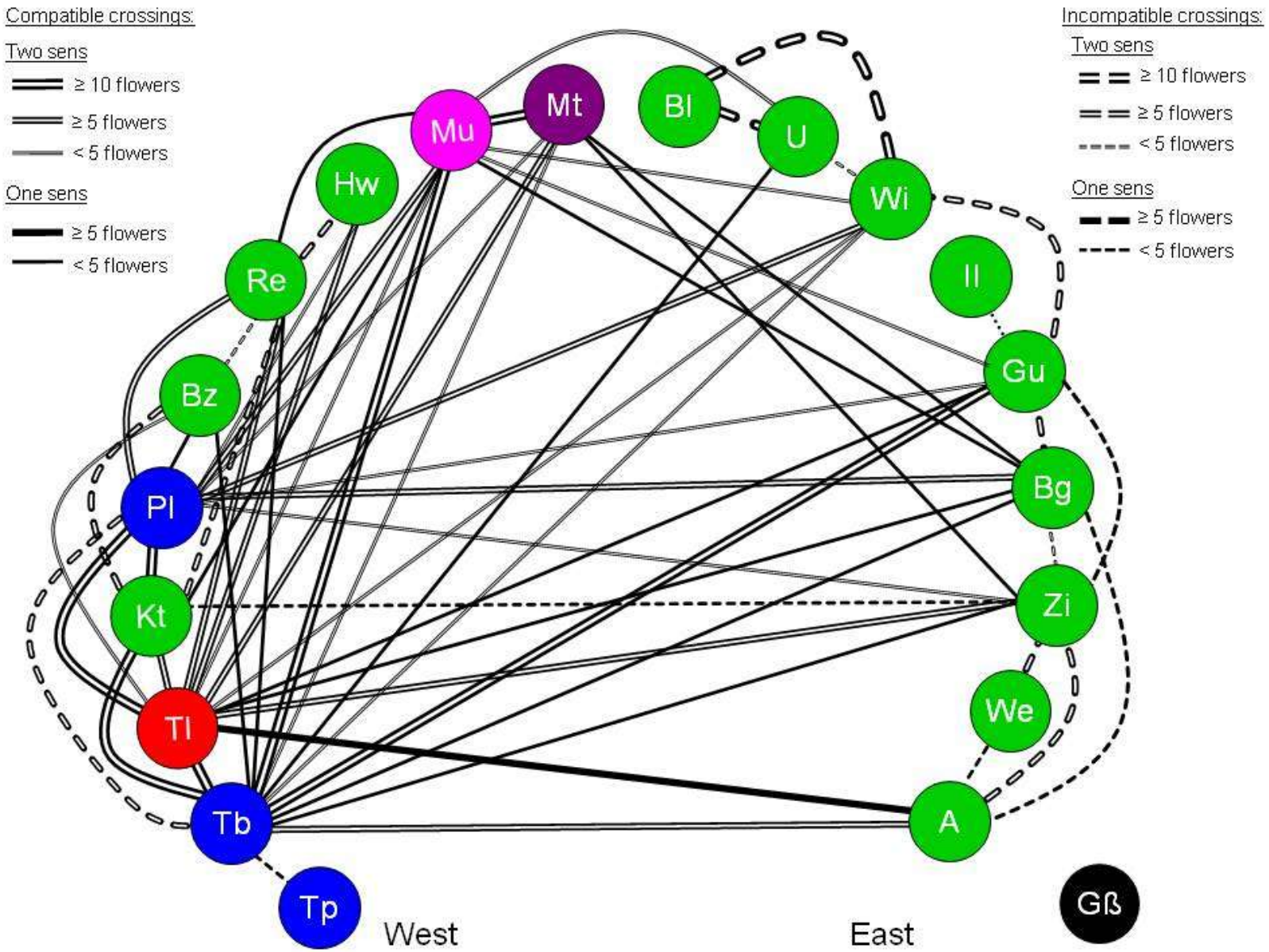
The unviable seeds correspond to the abnormal and the dead seeds together.

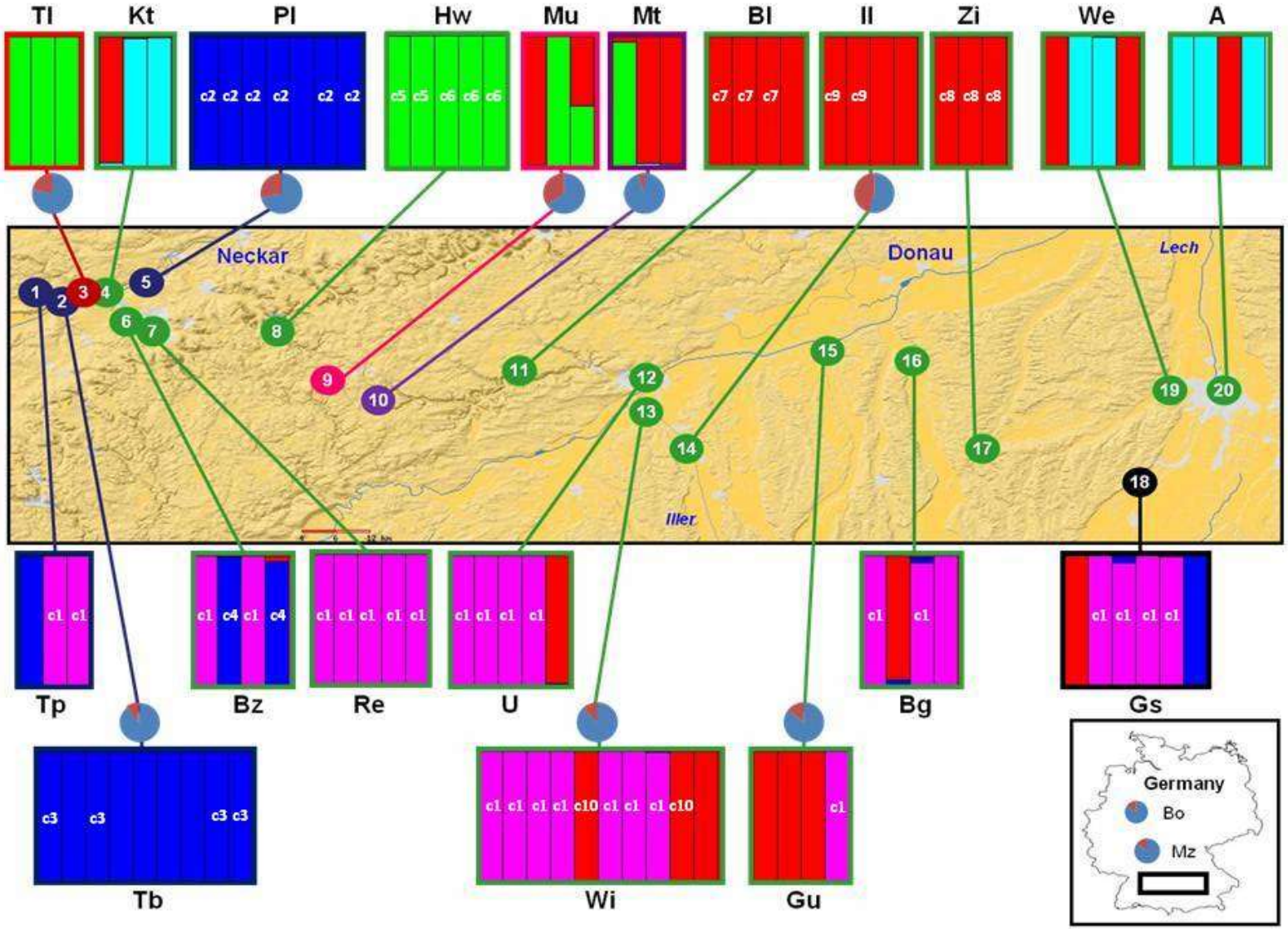
Appendix 11. Seed viability test within the “Tl-Pl-Re” triangle of the introduced area

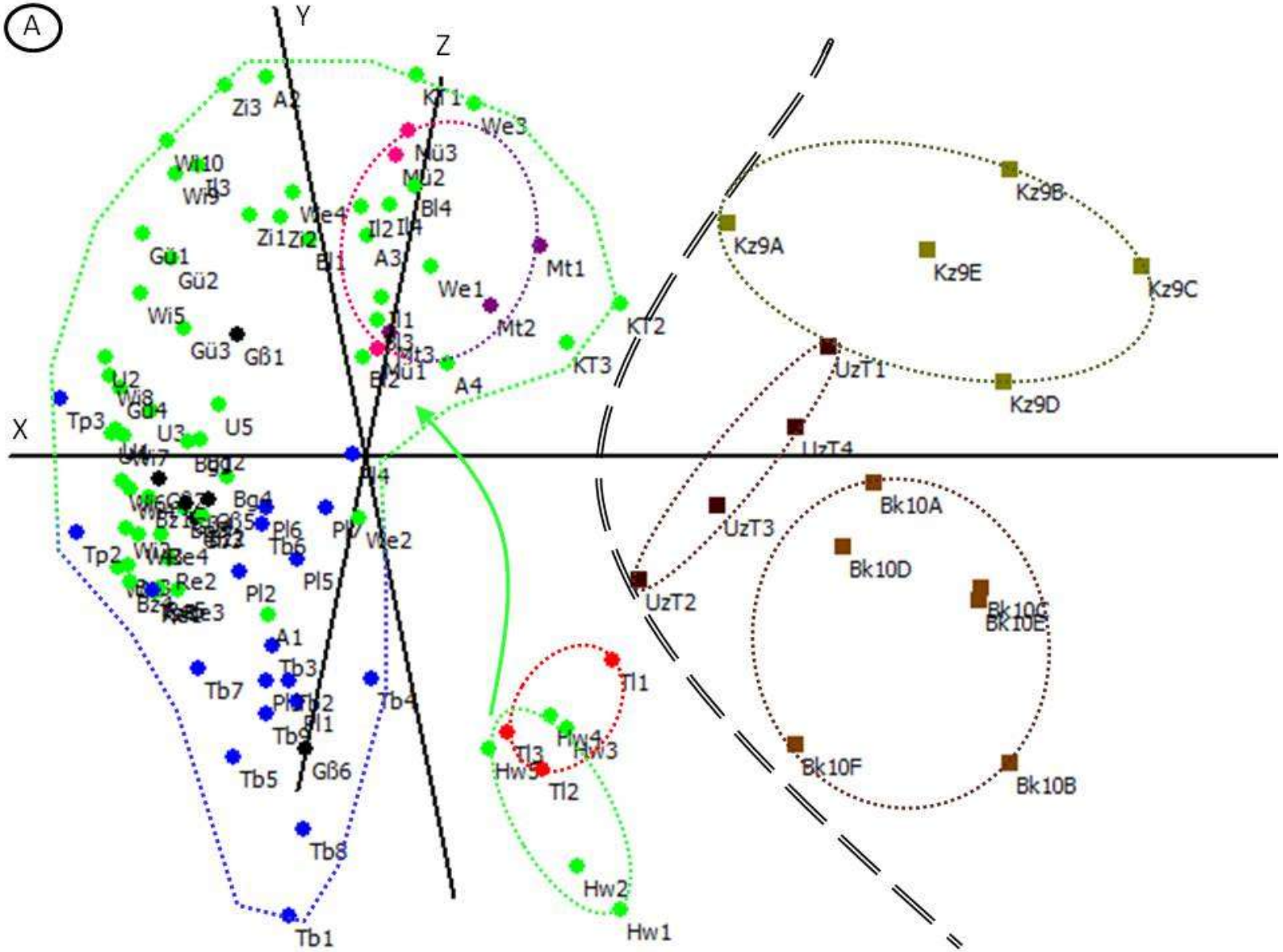
The colors correspond to the colors of the crossing groups (“Tl” population n°3 = red, “Pl” population n°5 = blue, “Re” population n°7 = green; Figure 3). Wilcoxon-Mann-Whitney-test compares the means of each observed characters for the three experiments: the means are

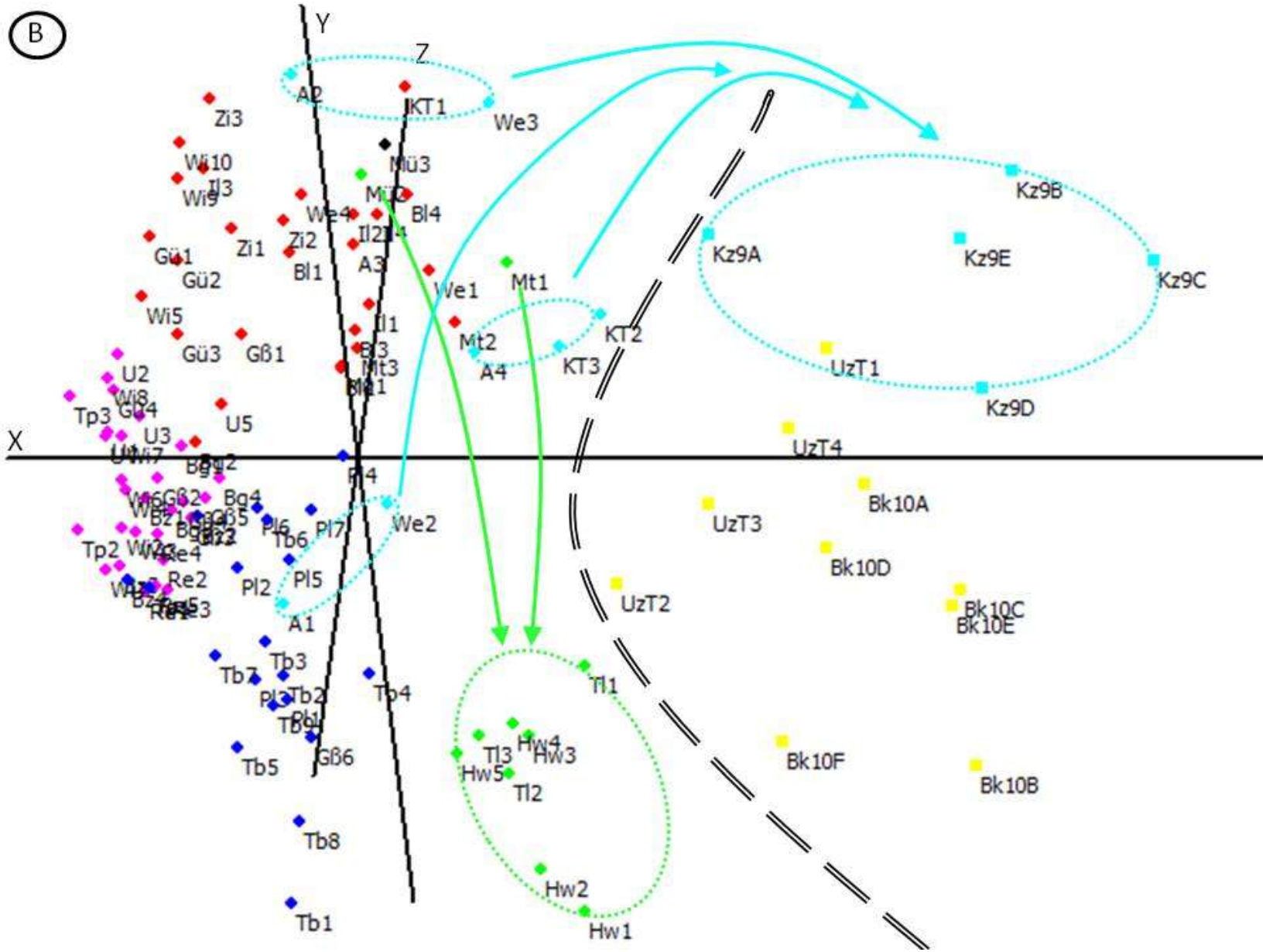
significantly different when the P-value is ≤ 0.05 (*), with a P-value ≤ 0.01 (**), with a P-value ≤ 0.001 (***) or are not significantly different when the P-value is > 0.05 (ns).
pop = population

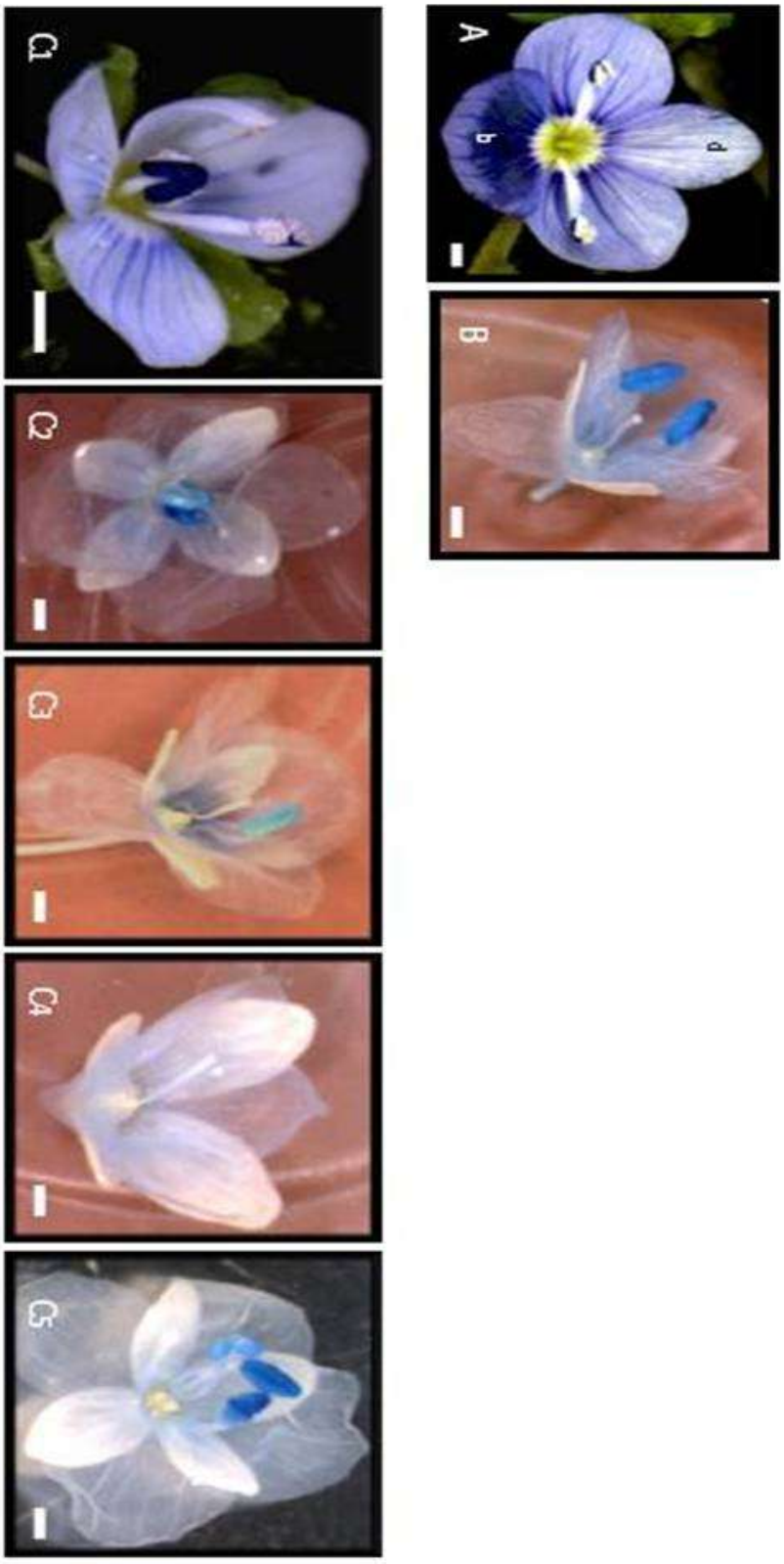

Bo
Mz
T-U-A
Kz
Bt
Lg
Bk
UzT
500 km

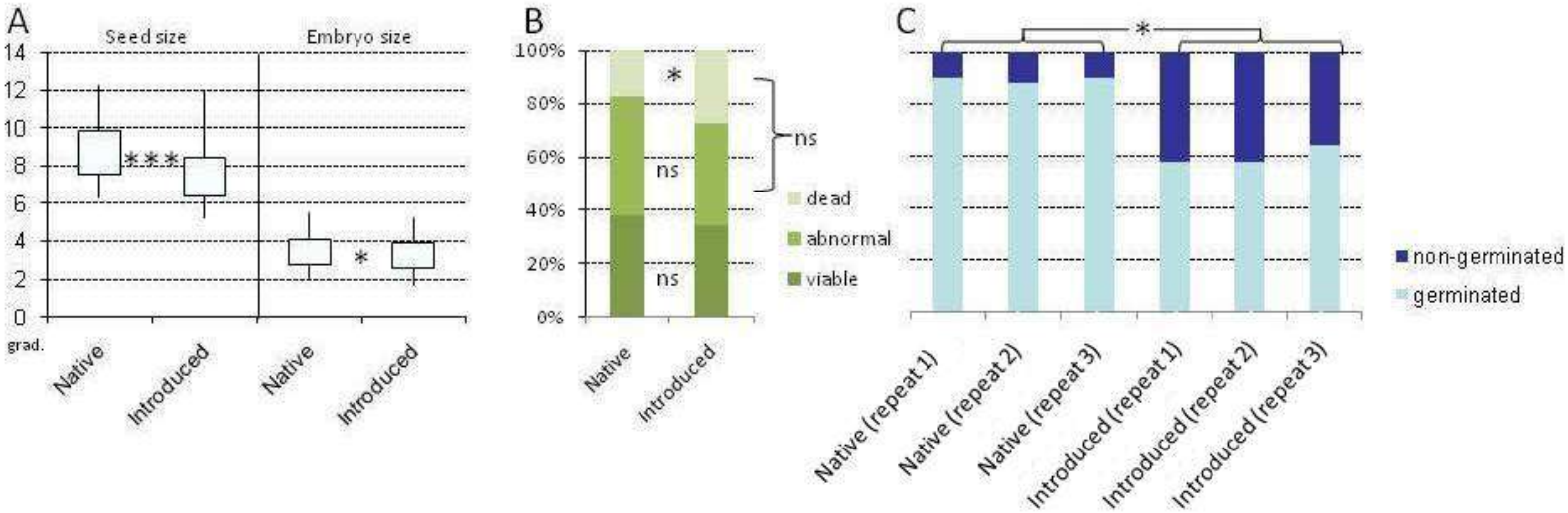

Compatible crossings:
Two sens
≥ 10 flowers
≥ 5 flowers
< 5 flowers
One sens
≥ 5 flowers
< 5 flowers
Incompatible crossings:
Two sens
≥ 10 flowers
≥ 5 flowers
< 5 flowers
One sens
≥ 5 flowers
< 5 flowers
Mt
Bl
Mu
U
Hw
Wi
Re
Il
Gu
Bz
Bg
Pl
Zi
Kt
We
Tl
A
Tb
Tp
West
East
Gß


Tl
Kt
Pl
Hw
Mu
Mt
Bl
Il
Zi
We
A
c2 c2 c2 c2 c2 c2
c5 c5 c6 c6 c6
c7 c7 c7
c9 c9
c8 c8 c8
Neckar
Donau
Lech
Iller
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
c1 c1
c1 c4 c1 c4
c1 c1 c1 c1 c1
c1 c1 c1 c1
c1 c1
c1 c1 c1 c1
Tp
Bz
Re
U
Bg
Gs
c3 c3 c3 c3
c1 c1 c1 c1 c10 c1 c1 c1 c10
c1
Tb
Wi
Gu
Germany
Bo
Mz


A
Y
Z
X
Zi3
A2
KT1
We3
Mü3
Mü2
Bl4
Wi10
Wi9
Il3
We4
Il2
Il4
Zi1
Zi2
Bl1
A3
Mt1
Gü1
Gü2
We1
Wi5
Il1
Mt2
KT2
Gü3
Gß1
Bl3
Mt3
Bl2
Mü1
A4
KT3
U2
Wi8
Gü4
Tp3
U3
U5
Bl4
Bg4
Pl6
Pl7
We2
Tb6
Tp2
Wi2
Re2
Pl5
Pl2
A1
Tb3
Tb7
Tb4
Pl1
Tb9
Gß6
Tb5
Tb8
Tb1
Tl1
Hw4
Hw3
Tl3
Hw5
Tl2
Hw2
Hw1
Kz9B
Kz9A
Kz9E
Kz9C
UzT1
Kz9D
UzT4
Bk10A
UzT3
Bk10D
UzT2
Bk10C
Bk10E
Bk10F
Bk10B


B
Y
Z
X
A2
KT1
We3
Zi3
Wi10
Il3
Wi9
Mü3
Mü2
We4
Bl4
Il2
Il4
Zi1
Zi2
Gü1
Bl1
A3
Gü2
We1
Mt1
Wi5
Il1
Mt2
KT2
Gü3
Gß1
Bl3
Mt3
KT3
A4
U2
Wi8
Bl1
Tp3
Gß4
U5
U3
Bg2
Pl4
Gß2
Bg4
Gß5
Pl6
Pl7
We2
Tb6
Tp2
Re2
Pl2
Pl5
A1
Tb3
Tb7
Pl3
Tb2
Tb4
Tb1
Tb5
Gß6
Tb8
Tl1
Hw4
Tl3
Hw3
Hw5
Tl2
Hw2
Hw1
Kz9B
Kz9A
Kz9E
Kz9C
UzT1
Kz9D
UzT4
Bk10A
UzT3
Bk10D
UzT2
Bk10C
Bk10E
Bk10F
Bk10B


A
a
b
c
d
B
C1
C2
C3
C4
C5


A
Seed size
Embryo size
14
12
10
8
6
4
2
0
grad.
***
*
Native
Introduced
Native
Introduced
B
100%
80%
60%
40%
20%
0%
*
ns
ns
ns
dead
abnormal
viable
Native
Introduced
C
*
non-germinated
germinated
Native (repeat 1)
Native (repeat 2)
Native (repeat 3)
Introduced (repeat 1)
Introduced (repeat 2)
Introduced (repeat 3)

Population	Area	Countries	Coordinates (N, E)		1_{st} obs.	Pop. Area	Habitat	$N_{AFLP\ (N\ clones)}$	Code
Uzungöl-Trabzon	Native	Turkey	N 40°37'18"	E 040°16'50"	---	large (<1600m²)	Plateau in the Anatolian mountains	4 (0)	UzT
Mleta	Native	Georgia	N 42°26'43"	E 044°29'15"	---	large (<1600m²)	Plateau in the Caucasian moutains (Greater)	---	Kz2
Goudaouri	Native	Georgia	N 42°28'30"	E 044°28'39"	---	large (<1600m²)	Plateau in the Caucasian moutains (Greater)	---	Kz4
Cross Pass	Native	Georgia	N 42°32'14"	E 044°28'38"	---	large (<1600m²)	Plateau in the Caucasian moutains (Greater)	---	Kz8
Kazbegi-Kobi	Native	Georgia	N 42°32'41"	E 044°29'28"	---	large (<1600m²)	Plateau in the Caucasian mountains (Greater)	5 (0)	Kz9
Bakuriani-Tskratskaro	Native	Georgia	N 41°38'17"	E 042°22'31"	---	large (<1600m²)	Plateau in the Caucasian mountains (Lesser)	6 (0)*	Bk10
Agara	Native	Georgia	N 41°41'40"	E 043°07'42"	---	large (<1600m²)	Plateau in the Caucasian moutains (Lesser)	---	Bk11
Khulo	Native	Georgia	N 41°38'17"	E 042°22'31"	---	large (<1600m²)	Plateau in the Caucasian moutains (Lesser)	---	Bt10
Chuasopeli	Native	Georgia	N 41°38'25"	E 042°27'30"	---	large (<1600m²)	Plateau in the Caucasian moutains (Lesser)	---	Bt11
Tübingen	Introduced	Germany BW	N 48°31'36"	E 009°02'11"	?	spot (<5m²)	Private garden, West Tübingen	3 (2)	Tp
Tübingen	Introduced	Germany BW	N 48°31'33"	E 009°05'12"	1909 [1]	spot (<5m²)	Old botanical garden, Central Tübingen	9 (4)	Tb
Tübingen	Introduced	Germany BW	N 48°31'26"	E 009°03'29"	1909 [1]	large (<750m²)	Private collective garden, East Tübingen	3 (0)	Tl
Kirchentellinsfurt	Introduced	Germany BW	N 48°32'01"	E 009°08'17"	?	spot (<5m²)	Grass close to a footpath	3 (0)	Kt
Pliezhausen	Introduced	Germany BW	N 48°33'03"	E 009°11'02"	1988 [2]	large (<1600m²)	Grass close to a footpath	7 (6)	Pl
Betzingen	Introduced	Germany BW	N 48°29'58"	E 009°10'17"	?	intermediary (<25m²)	Old cemetery of Reutlingen	4 (4)*	Bz
Reutlingen	Introduced	Germany BW	N 48°28'38"	E 009°11'09"	1987 [2]	large (<1600m²)	Parc of Schlattwiesen	5 (5)*	Re
Hohenwittlingen	Introduced	Germany BW	N 48°28'17"	E 009°25'18"	1984 [2]	spot (<5m²)	Fruit tree culture, East Bad Urach	5 (5)***	Hw
Münsingen	Introduced	Germany BW	N 48°24'39"	E 009°29'28"	2001 [2]	intermediary (<25m²)	Grass close to the train station	3 (0)	Mü
Mehrstetten	Introduced	Germany BW	N 48°22'31"	E 009°33'47"	2001 [2]	large (<300m²)	Private garden	3 (0)	Mt
Blaubeuren	Introduced	Germany BW	N 48°24'52"	E 009°47'24"	1975 [2]	spot (<5m²)	Grass close to a school	4 (3)*	Bl
Ulm	Introduced	Germany BV	N 48°23'38"	E 009°59'16"	2003 [2]	large (<500m²)	Old Botanical Garden of Ulm	5 (3)	U
Wiblingen	Introduced	Germany BV	N 48°21'18"	E 009°59'04"	1936 [1]	large (<500m²)	Old cemetery of Ulm	10 (9)*	Wi
Illerzell	Introduced	Germany BV	N 48°17'44"	E 010°03'24"	2003 [2]	large (<500m²)	Private collective garden	4 (2)	Il
Günzburg	Introduced	Germany BV	N 48°27'14"	E 010°16'38"	?	spot (<5m²)	Public garden in front of the castel	4 (1)	Gü
Burgau	Introduced	Germany BV	N 48°25'32"	E 010°25'08"	?	intermediary (<25m²)	Private garden	4 (2)*	Bg
Ziemetshausen	Introduced	Germany BV	N 48°17'28"	E 010°31'54"	?	spot (<5m²)	Private garden	3 (3)	Zi
Großaitingen	Introduced	Germany BV	N 48°13'56"	E 010°46'51"	?	spot (<5m²)	Grass close to a footpath	6 (4)	Gß
Westheim	Introduced	Germany BV	N 48°23'02"	E 010°48'59"	?	intermediary (<25m²)	Grass close to a school	4 (0)	We
Augsburg	Introduced	Germany BV	N 48°22'38"	E 010°54'01"	1939 [1]	intermediary (<25m²)	Public garden, Stephingergraben	4 (0)	A
Bonn-Rheinaue	Introduced	Germany RW	N 50°42'44"	E 007°08'17"	?	large (<1600m²)	Public garden along the Rhein river	---	Bo
Mainz	Introduced	Germany RP	N 49°59'24"	E 008°14'22"	?	intermediary (<25m²)	Arboretum, Botanical Garden	---	Mz

		n_{pop}	$N_{samples}$	Frag.	% Frag.	Frag.	Frag.	Hj	S. D.	Var.
		Code		polym.	polym.	fixed	priv.			
All		23	108	261	88,78%	4	---	0.08023	0.03449	0.001190
Native area		3	15	158	53,74%	8	46	0.13702	0.02739	0.000750
Locality	Region									
Uzungöl-Trabzon	*Turkey-Eastern*	UzT	4	72	24,49%	35	8	0.12754	0.01326	0.000176
Kazbegi-Kobi	*Georgia-Northern*	Kz9	5	97	32,99%	11	17	0.16790	0.01460	0.000213
Bakuriani-Tskratskaro	*Georgia-Central*	Bk10	6	80	27,21%	42	13	0.11564	0.01154	0.000133
Introduced area		20	93	213	72,45%	5	98	0.07171	0.02670	0.000713
Locality	Crossing group									
Tübingen	*blue*	Tp	3	24	8,16%	61	1	0.04488	0.00923	0.000085
Tübingen	*blue*	Tb	9	60	20,41%	45	3 (1)	0.07510	0.00935	0.000087
Tübingen	*red*	Tl	3	33	11,22%	70	0	0.06377	0.01087	0.000118
Kirchentellinsfurt	*green*	Kt	3	62	21,09%	26	3	0.12550	0.01419	0.000201
Pliezhausen	*blue*	Pl	7	52	17,69%	51	2	0.06997	0.00944	0.000089
Betzingen	*green*	Bz	4	24	8,16%	69	3 (1)	0.05867	0.00959	0.000092
Reutlingen	*green*	Re	5	20	6,80%	71	1	0.03476	0.00697	0.000049
Hohenwittlingen	*green*	Hw	5	31	10,54%	75	0	0.06321	0.01005	0.000101
Münsingen	*pink*	Mü	3	34	11,56%	52	1	0.06200	0.01125	0.000126
Mehrstetten	*violet*	Mt	3	27	9,18%	66	3	0.05312	0.00998	0.000100
Blaubeuren	*green*	Bl	4	34	11,56%	55	6	0.06675	0.01022	0.000104
Ulm	*green*	U	5	43	14,63%	47	0	0.06364	0.00931	0.000087
Wiblingen	*green*	Wi	10	45	15,31%	45	0	0.05465	0.00807	0.000065
Illerzell	*green*	Il	4	39	13,27%	45	3 (1)	0.06526	0.00982	0.000096
Günzburg	*green*	Gü	4	48	16,33%	42	3	0.07981	0.01078	0.000116
Burgau	*green*	Bg	4	32	10,88%	63	2	0.05919	0.00983	0.000097
Ziemetshausen	*green*	Zi	3	37	12,59%	42	3	0.06627	0.01157	0.000134
Großaitingen	*unknown*	Gß	6	49	16,67%	47	6	0.06514	0.00895	0.000080
Westheim	*green*	We	4	75	25,51%	23	6	0.13030	0.01316	0.000173
Augsburg	*green*	A	4	73	24,83%	32	7	0.13221	0.01445	0.000209
	all green	13	59	185	62,93%	7	47	0.07694	0.03151	0.000993
	all blue	3	19	87	29,59%	35	7	0.06332	0.01617	0.000261
	other	4	15	102	34,69%	30	10	0.07171	0.02670	0.000713

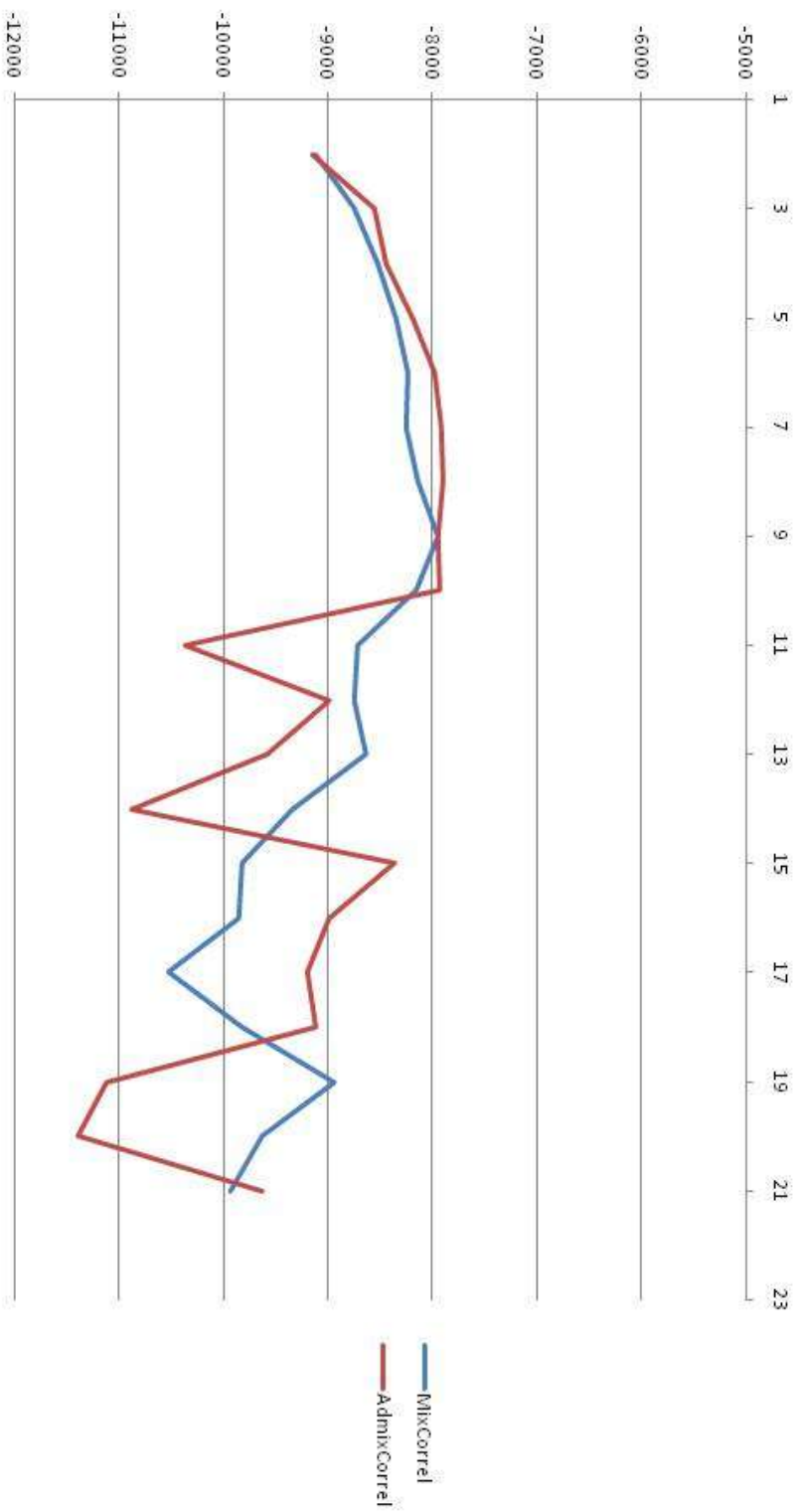
Sampling	Grouping (code)	N	source of variation	d.f.	SS	Variance components	Percentage of variance	Fixation index
All	***Area***	2	Among areas	1	1.15	0.041	32.64%	0.326
	(Nat. / Intr.)		Within areas	106	9.05	0.085	67.35%	
					V_{total} =	0.126		
Native area	***Region***	2	Among regions	1	0.25	0.015	9.27%	0.093
	(Anat. / G+L Cauc.)		Within regions	13	2.28	0.156	90.73%	
					V_{total} =	0.172		
	Population	3	Among populations	2	0.705	0.044	25.48%	0.255
	(UzT / Kz9 / Bk10)		Within populations	12	1.575	0.131	74.52%	
					V_{total} =	0.176		
Introduced area	***Population***	20	Among populations	19	3.779	0.034	45.47%	0.455
	(20 transect pop.)		Within populations	73	2.999	0.041	54.53%	
					V_{total} =	0.075		
	Crossing group	5	Among crossing groups	4	0.905	0.014	18.16%	0.182
	(g / b / r / p / v)		Within crossing groups	82	5.527	0.067	81.84%	
					V_{total} =	0.082		
	Genetic group	6	Among genetic groups	5	1.527	0.021	26.09%	0.261
	(P / T / B / Y / G / R)		Within genetic groups	93	5.252	0.059	73.90%	
					V_{total} =	0.080		

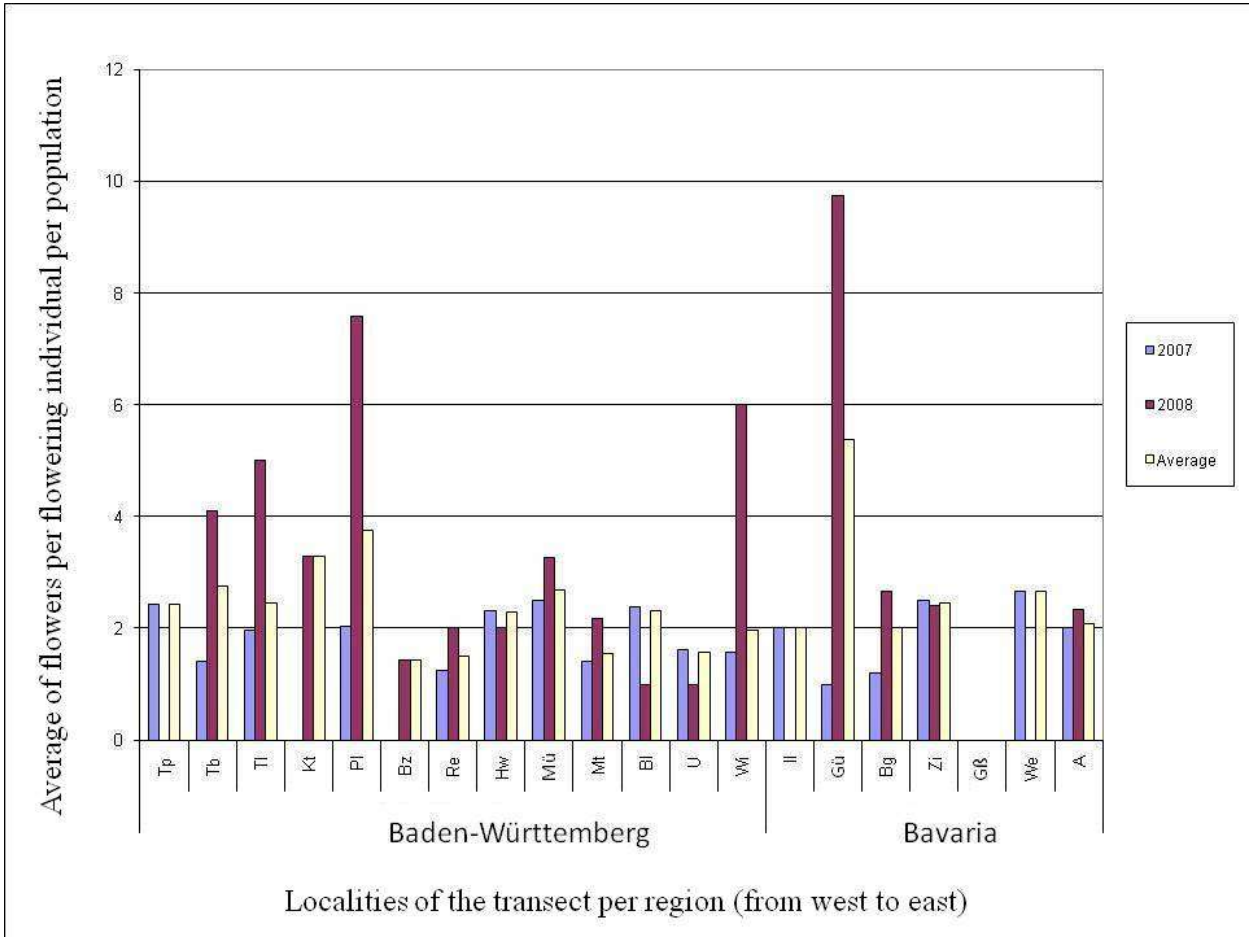
Area	Region	Population	Code	$N_{flowers}$	Pollen	S.D.	% S.D.	Ovule	S.D.	% S.D.	Cros. group	Gen. group
Native			Nat.	43	11500	2309	20,10%	11,6	0,9	8,00%	---	---
	Kazbegi		GC	30	11160	4512	40,43%	11,9	1,4	12,04%	---	---
		Mleta	Kz2	10	13060	4471	34,20%	12,4	1,3	10,20%	---	---
		Cross Pass	Kz8	10	7610	4097	53,80%	11,8	1,8	14,80%	---	---
		Kazbegi-Kobi	Kz9	10	12810	2788	21,80%	11,6	1,3	10,90%	---	---
	Adjara		LC	13	12259	5248	42,81%	11,1	1,8	15,87%	---	---
		Khulo	Bt10	5	11140	3035	27,20%	12,0	0,0	0,00%	---	---
		Chuasopeli	Bt11	8	12881	4223	34,20%	10,0	2,3	23,10%	---	---
Introduced			Intr.	92	7988	2350	29,40%	12,0	1,8	14,60%	4	4
	Baden-Württemberg		BW	48	7102	3610	50,82%	12,0	2,3	18,88%	---	---
		Tübingen	Tb	5	10658	2507	23,50%	9,3	1,7	17,90%	blue	Blue
		Pliezhausen	Pl	15	4641	2405	51,80%	10,2	1,5	15,00%	blue	Blue
		Reutlingen	Re	10	4840	2459	50,80%	13,0	1,3	10,30%	green	Pink
		Münsingen	Mü	8	9900	3874	39,10%	10,9	1,2	11,50%	pink	Green / Red
		Mehrstetten	Mt	10	6950	3194	46,00%	14,2	2,7	19,30%	violet	Red
	Bavaria		BV	44	9080	2514	27,68%	13,1	1,4	10,65%	---	---
		Wiblingen	Wi	10	8578	2604	30,40%	13,4	1,9	14,30%	green	Pink
		Illerzell	Il	9	8035	2720	33,90%	13,5	2,1	15,70%	green	Red
		Günzburg	Gü	25	10299	2393	23,20%	12,9	1,1	8,10%	green	Red

Area / Region	Population	Code	$N_{caps.}$	$N_{Seeds/Caps.}$	S.D.	% S.D.	Total N_{Seed}	Length	Width	Cros. group	Gen. group
Native		Nat.	53	6.3	2.4	38.17%	472	1.34	0.93	---	---
Greater Caucasus		GC	28	6.6	2.3	34.85%	237	1.33	0.93	---	---
Kakheti	Telavi	Lg1	---	---	---	---	38	1.11	0.82	---	---
	Tetritslebi	Lg3	6	6.0	2.4	40.00%	36	1.38	0.96	---	---
	Telavi-Gombori A	Lg4	5	8.0	1.2	15.00%	43	1.46	1.05	---	---
	Telavi-Gombori B	Lg5	1	5.0	0.0	0.00%	---	---	---	---	---
	Telavi-Gombori C	Lg6	3	7.0	1.7	24.29%	29	1.39	1.01	---	---
	Telavi-Gombori D	Lg7	11	6.5	2.7	41.54%	78	1.33	0.87	---	---
	Telavi-Gombori E	Lg8	2	6.5	3.5	53.85%	13	1.30	0.99	---	---
Lesser Caucasus		LC	25	6.0	2.6	42.67%	235	1.34	0.93	---	---
Adjara	Batumi-Batskana	Bt1	---	---	---	---	36	1.39	1.00	---	---
	Khulo	Bt10	6	7.7	1.2	15.71%	59	1.39	0.95	---	---
	Chuasopeli	Bt11	2	7.0	0.0	0.00%	14	1.44	0.92	---	---
	Batumi	Bt12	1	10.0	0.0	0.00%	---	---	---	---	---
Samtskhe-	Borjomi	Bk2	7	4.9	2.4	49.18%	34	1.47	1.08	---	---
Javakhéti	Tsagueri, r. Gujarula	Bk3	3	4.0	2.6	66.00%	---	---	---	---	---
	Tsagueri	Bk4	4	4.3	2.6	61.16%	17	1.35	0.95	---	---
	Pataratsemi	Bk5	2	8.5	0.7	8.35%	17	1.20	0.83	---	---
	Bakuriani A	Bk7	---	---	---	---	43	1.10	0.74	---	---
	Bakuriani B	Bk8	---	---	---	---	15	1.47	0.97	---	---
Introduced		Intr.	83	7.4	2.8	37.33%	690	1.12	0.83	---	---
Baden-Württemberg		BW	75	7.5	2.7	36.17%	620	1.12	0.82	---	---
	Tübingen	Tb	5	7.2	1.3	18.06%	42	0.93	0.65	blue	Blue
	Tübingen	Tl	26	9.3	2.1	22.14%	246	1.12	0.80	red	Green
	Kirchentellinsfurt	Kt	8	8.3	2.6	31.52%	66	1.15	0.83	green	Turquoise
	Pliezhausen	Pl	18	5.5	2.3	42.36%	99	1.20	0.89	blue	Blue
	Reutlingen	Re	4	7.3	1.9	26.07%	29	0.97	0.71	green	Pink
	Münsingen	Mü	4	7.3	3.3	45.52%	52	1.14	0.82	pink	Green / Red
	Mehrstetten	Mt	10	6.4	3.0	47.27%	86	1.17	0.91	violet	Red
Bavaria		BV	8	6.5	3.3	50.00%	70	1.11	0.90	---	---
	Ulm	U	1	2.0	0.0	0.00%	---	---	---	green	Pink
	Wiblingen	Wi	2	3.5	0.7	20.20%	10	0.99	0.79	green	Pink
	Burgau	Bg	2	9.0	0.0	0.00%	18	1.00	0.83	green	Pink
	Ziemetshausen	Zi	2	9.5	2.1	22.32%	30	1.17	0.95	green	Red
	Augsburg	A	1	6.0	0.0	0.00%	12	1.24	0.95	green	Turquoise

Area	Region	Population	Code	N_{embryo}	Embryo	S.D.	% S.D.	Cros. group	Gen. Group
Native			Nat.	137	3,44	0,69	20,02%	---	---
	Kakheti	6 pop.	Lg 1/3/4/6/7/8	47	3,55	0,68	19,26%	---	---
	Adjara	3 pop.	Bt1/10/11	47	3,46	0,71	20,58%	---	---
	Samtskhe- Javakhéti	5 pop.	Bk2/4/5/7/8	43	3,31	0,69	20,79%	---	---
Introduced			Intr.	132	3,25	0,69	21,14%	---	---
	Baden-Württemberg	Tübingen	Tl	42	3,10	0,64	20,73%	red	Green
	Baden-Württemberg	Pliezhausen	Pl	47	3,67	0,66	18,03%	blue	Blue
	Baden-Württemberg	Reutlingen	Re	43	2,95	0,53	18,10%	green	Pink

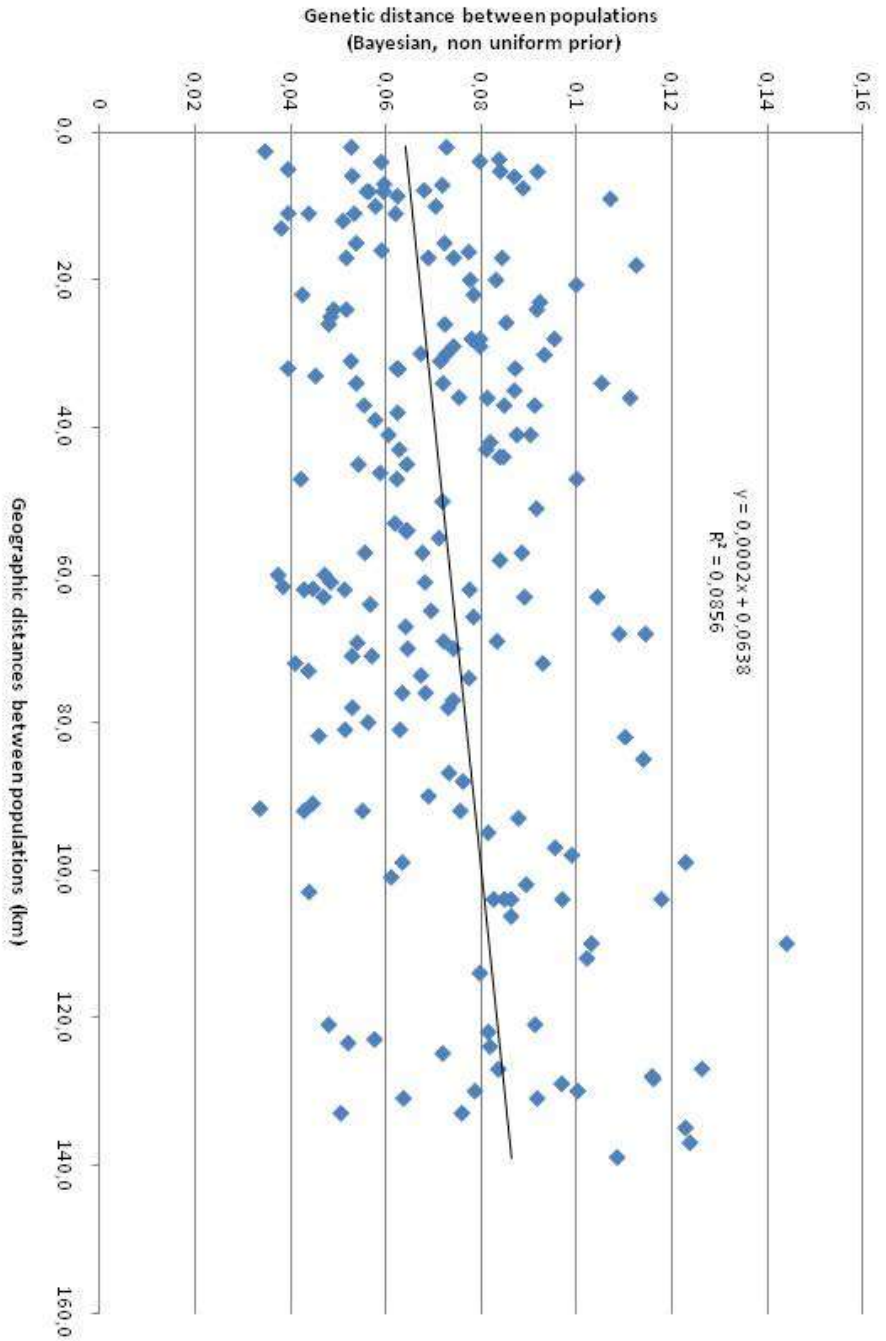
Population	Region	Mountains	Code	DNA	Flower	Bud	Capsule	Seed	Coordinates (N,E)	
Mleta	Kazbegi	Greater Caucasus	Kz2		91 (1)	10			N 42°26'43"	E 044°29'15"
Goudaouri	Kazbegi	Greater Caucasus	Kz4		254 (0)				N 42°28'30"	E 044°28'39"
Cross Pass	Kazbegi	Greater Caucasus	Kz8		202 (6)	10			N 42°32'14"	E 044°28'38"
Kazbegi-Kobi	Kazbegi	Greater Caucasus	Kz9	5	189 (150)	10			N 42°32'58"	E 044°29'40"
Telavi	Kakheti	Greater Caucasus	Lg1					38	N 41°54'26"	E 045°22'45"
Tetritslebi	Kakheti	Greater Caucasus	Lg3				6	36	N 41°51'45"	E 045°19'02"
Cross Pass Telavi-Gombori A	Kakheti	Greater Caucasus	Lg4				5	43	N 41°52'08"	E 045°17'00"
Cross Pass Telavi-Gombori B	Kakheti	Greater Caucasus	Lg5				1		N 41°52'12"	E 045°18'18"
Cross Pass Telavi-Gombori C	Kakheti	Greater Caucasus	Lg6				3	29	N 41°52'17"	E 045°20'53"
Cross Pass Telavi-Gombori D	Kakheti	Greater Caucasus	Lg7				11	78	N 41°52'40"	E 045°16'43"
Cross Pass Telavi-Gombori E	Kakheti	Greater Caucasus	Lg8				2	13	N 41°52'30"	E 045°14'56"
Borjomi	Samtskhe-Javakhéti	Lesser Caucasus	Bk2				7	34	N 41°48'45"	E 043°26'25"
Tsagueri, river Gujarula	Samtskhe-Javakhéti	Lesser Caucasus	Bk3				3		N 41°48'25"	E 043°28'09"
Tsagueri	Samtskhe-Javakhéti	Lesser Caucasus	Bk4				4	17	N 41°47'48"	E 043°28'20"
Pataratsemi	Samtskhe-Javakhéti	Lesser Caucasus	Bk5				2	17	N 41°47'40"	E 043°28'05"
Bakuriani A	Samtskhe-Javakhéti	Lesser Caucasus	Bk7					43	N 41°44'09"	E 043°31'03"
Bakuriani B	Samtskhe-Javakhéti	Lesser Caucasus	Bk8					15	N 41°43'54"	E 043°30'21"
Bakuriani-Tskratskaro	Samtskhe-Javakhéti	Lesser Caucasus	Bk10	6					N 41°41'30"	E 043°31'11"
Agara	Samtskhe-Javakhéti	Lesser Caucasus	Bk11		1174 (24)				N 41°41'40"	E 043°07'42"
Batumi-Batskana	Adjara	Lesser Caucasus	Bt1					36	N 41°39'01"	E 041°40'02"
Khulo	Adjara	Lesser Caucasus	Bt10		171 (1)	5	6	59	N 41°38'17"	E 042°22'31"
Chuasopeli	Adjara	Lesser Caucasus	Bt11		55 (0)	8	2	14	N 41°38'25"	E 042°27'30"
Batumi	Adjara	Lesser Caucasus	Bt12				1		N 41°38'01"	E 042°29'49"

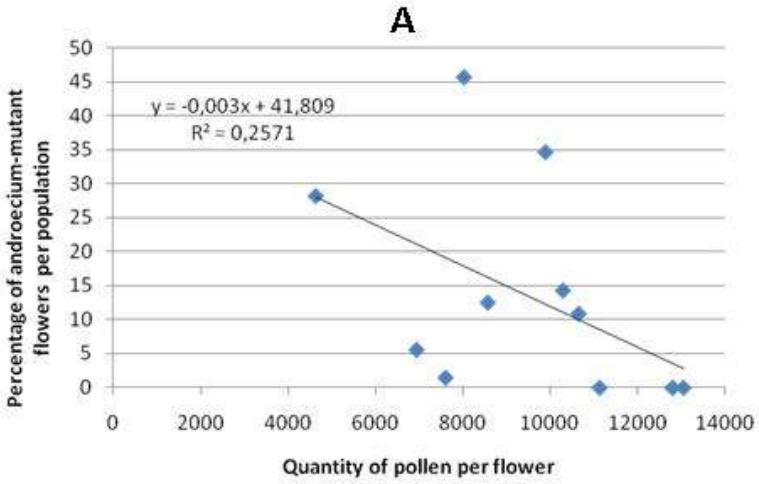

-5000
-6000
-7000
-8000
-9000
-10000
-11000
-12000
1
3
5
7
9
11
13
15
17
19
21
23
MixCorrel
AdmixCorrel

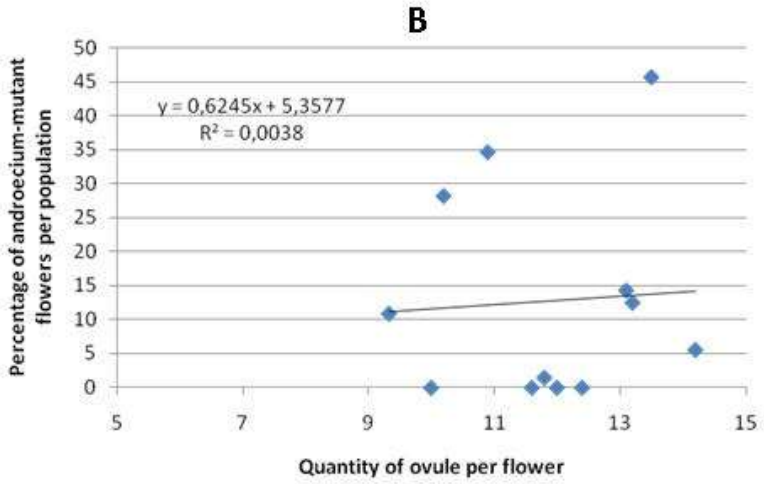

Average of flowers per flowering individual per population
12
10
8
6
4
2
0
Tp
Tb
Tl
Kt
Pl
Bz
Re
Hw
Mü
Mt
Bl
U
Wi
Il
Gü
Bg
Zi
Gß
We
A
Baden-Württemberg
Bavaria
2007
2008
Average
Localities of the transect per region (from west to east)

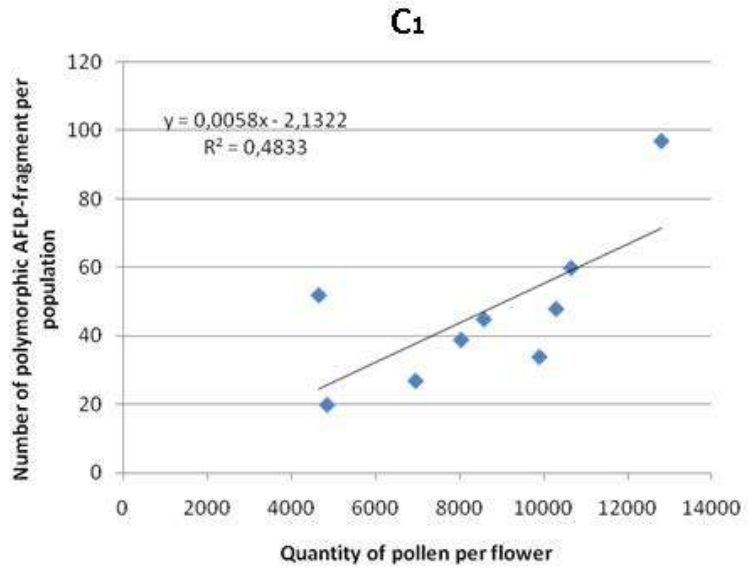
A) Source of variation	Pollen number		
	Df	**F**	**p-value**
Area	1	29.489	P<0.000***
within native			
Region	1	0.491	P=0.534
Population	4	6.263	P=0.0015**
without Kz8	3	1.248	P=0.3019
within introduced			
Region	1	2.139	P=0.2387
Population	7	8.252	P<0.000***
Crossing group	3	2.481	P=0.066
Genetic group	3	3.519	p=0.018*

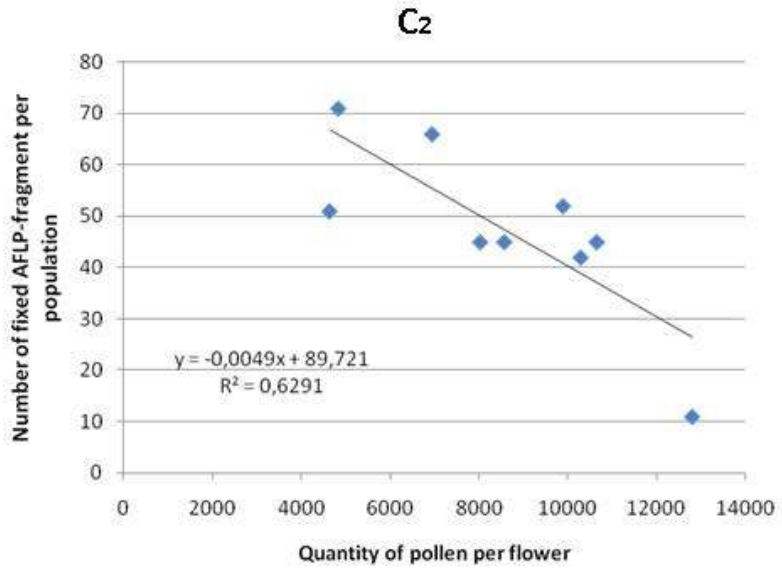
B) Source of variation	Ovule number			Seed number			Seed size			Embryo size		
	Df	H	p-value	Df	H	p-value	Df	H	p-value	Df	H	p-value
Area	1	4.899	P=0.027*	1	5.064	P=0.024*	1	265.052	P<0.000***	1	4.472	P=0.034*
within native												
Region	1	1.753	P=0.185	1	1.016	P=0.313	1	0.748	P=0.387	1	3.964	P=0.130
Population	4	5.730	P=0.220	12	17.786	P=0.122	13	169.202	P<0.000***	--	---	---
within introduced												
Region	1	7.794	P=0.005**	1	0.677	P=0.411	1	0.001	P=0.983	--	---	---
Population	7	29.651	P<0.001***	11	32.428	P<0.001***	11	95.026	P<0.001***			
Crossing group	3	27.604	P<0.001***	4	21.597	P<0.000***	4	11.775	P=0.019*	2	25.311	P<0.000***
Genetic group	3	18.211	P<0.001***	4	22.353	p<0.000***	4	41.598	P<0.001***			

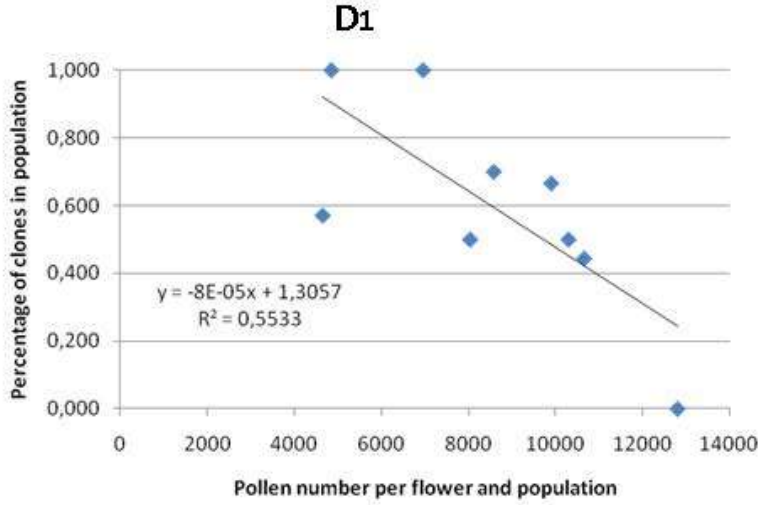

Genetic distance between populations
(Bayesian, non uniform prior)
0
0,02
0,04
0,06
0,08
0,1
0,12
0,14
0,16
y = 0,0002x + 0,0638
R² = 0,0856
0,0
20,0
40,0
60,0
80,0
100,0
120,0
140,0
160,0
Geographic distances between populations (km)

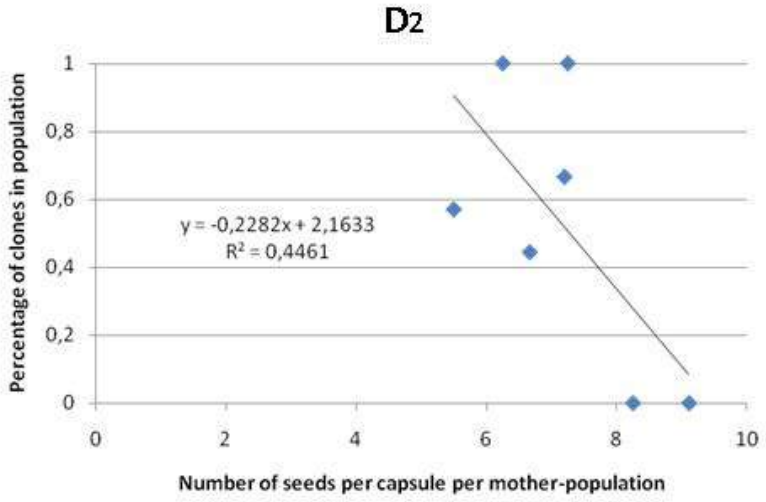

A
y = -0,003x + 41,809
R² = 0,2571
Percentage of androecium-mutant flowers per population
Quantity of pollen per flower

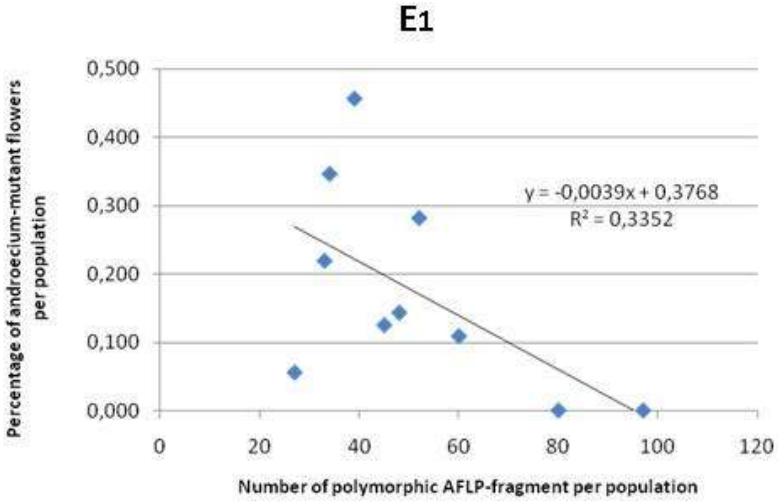

B
y = 0,6245x + 5,3577
R² = 0,0038
Percentage of androecium-mutant flowers per population
Quantity of ovule per flower

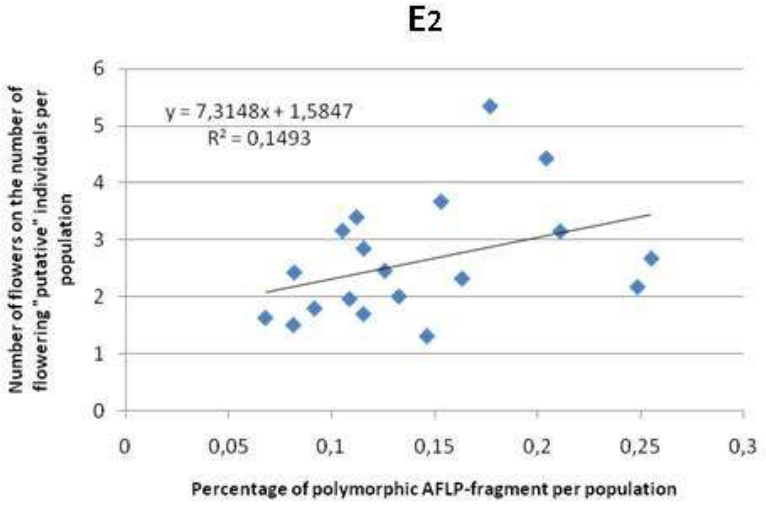

C1
y = 0,0058x - 2,1322
R² = 0,4833
Number of polymorphic AFLP-fragment per population
Quantity of pollen per flower

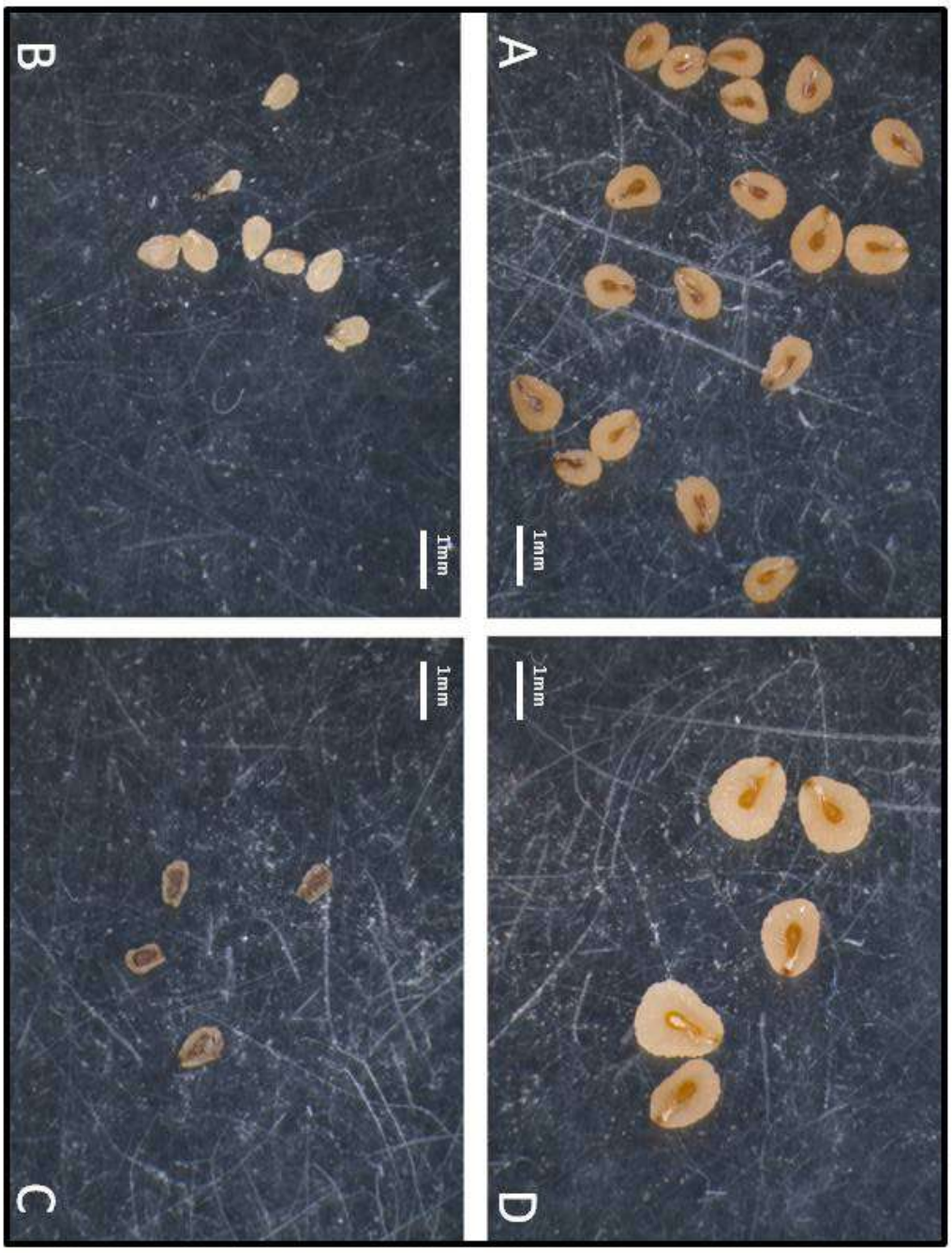

C2
y = -0,0049x + 89,721
R² = 0,6291
Number of fixed AFLP-fragment per population
Quantity of pollen per flower

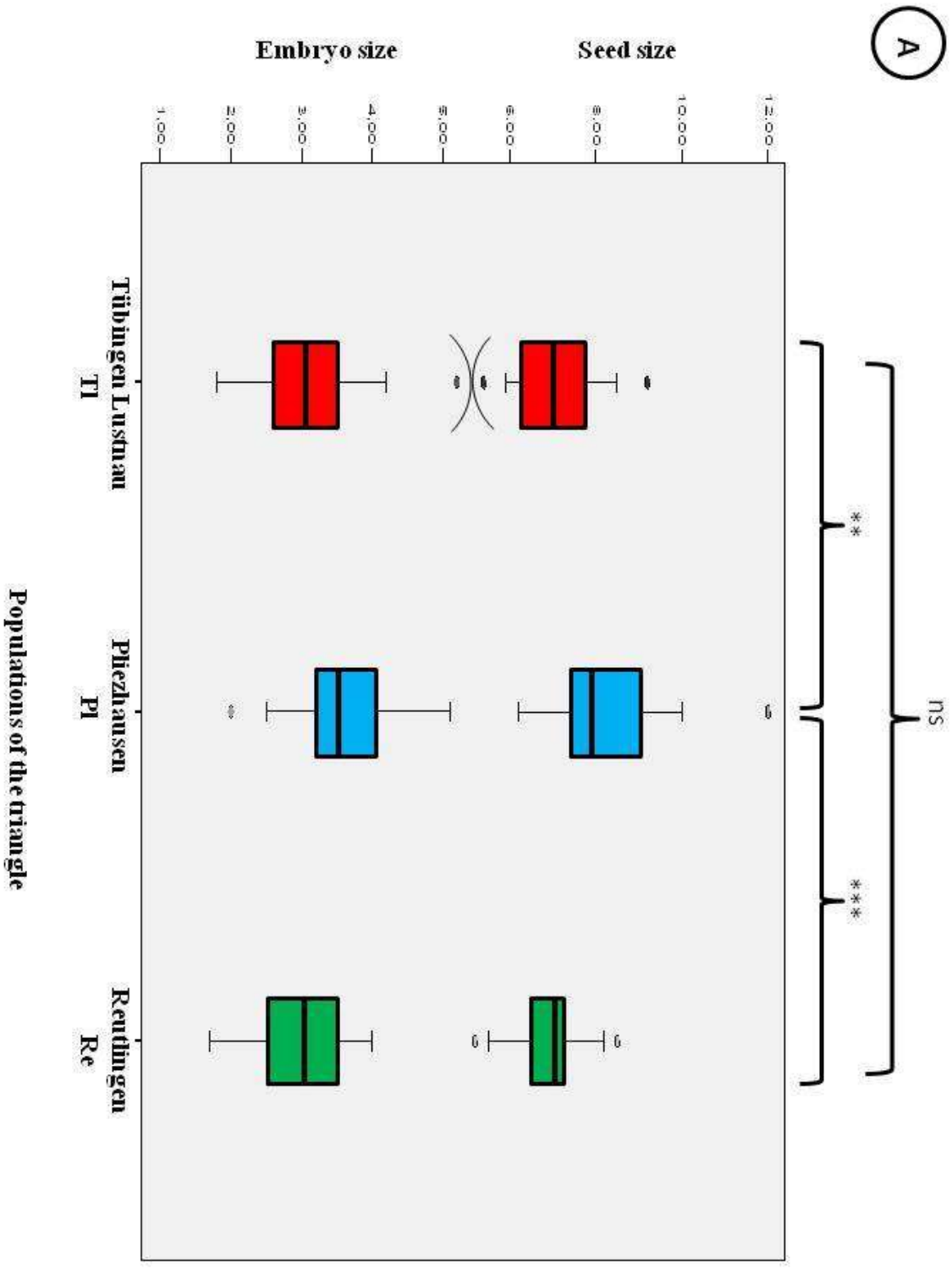

D1
Percentage of clones in population
1,000
0,800
0,600
0,400
0,200
0,000
y = -8E-05x + 1,3057
R² = 0,5533
0
2000
4000
6000
8000
10000
12000
14000
Pollen number per flower and population

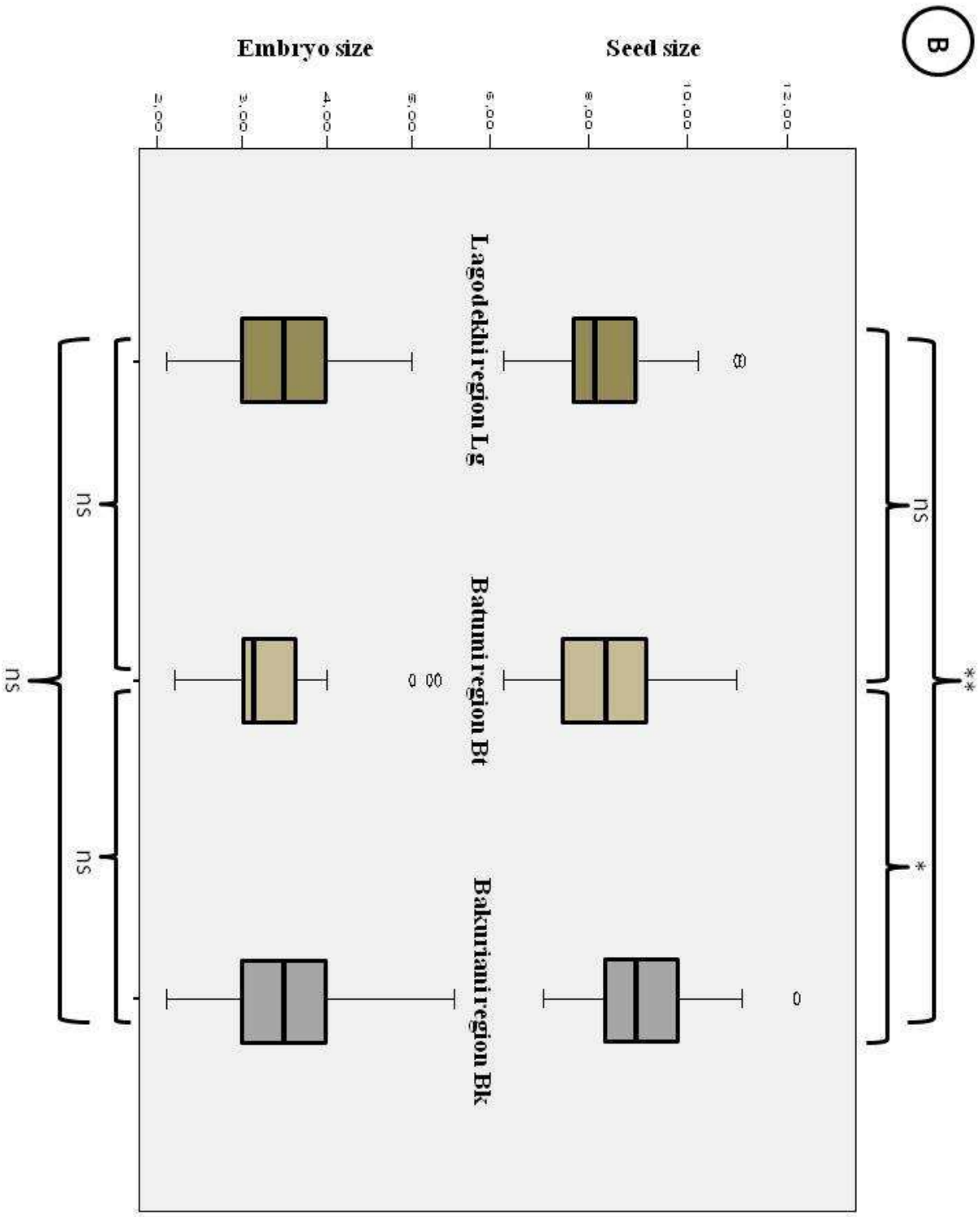

D2
Percentage of clones in population
1
0,8
0,6
0,4
0,2
0
y = -0,2282x + 2,1633
R² = 0,4461
0
2
4
6
8
10
Number of seeds per capsule per mother-population

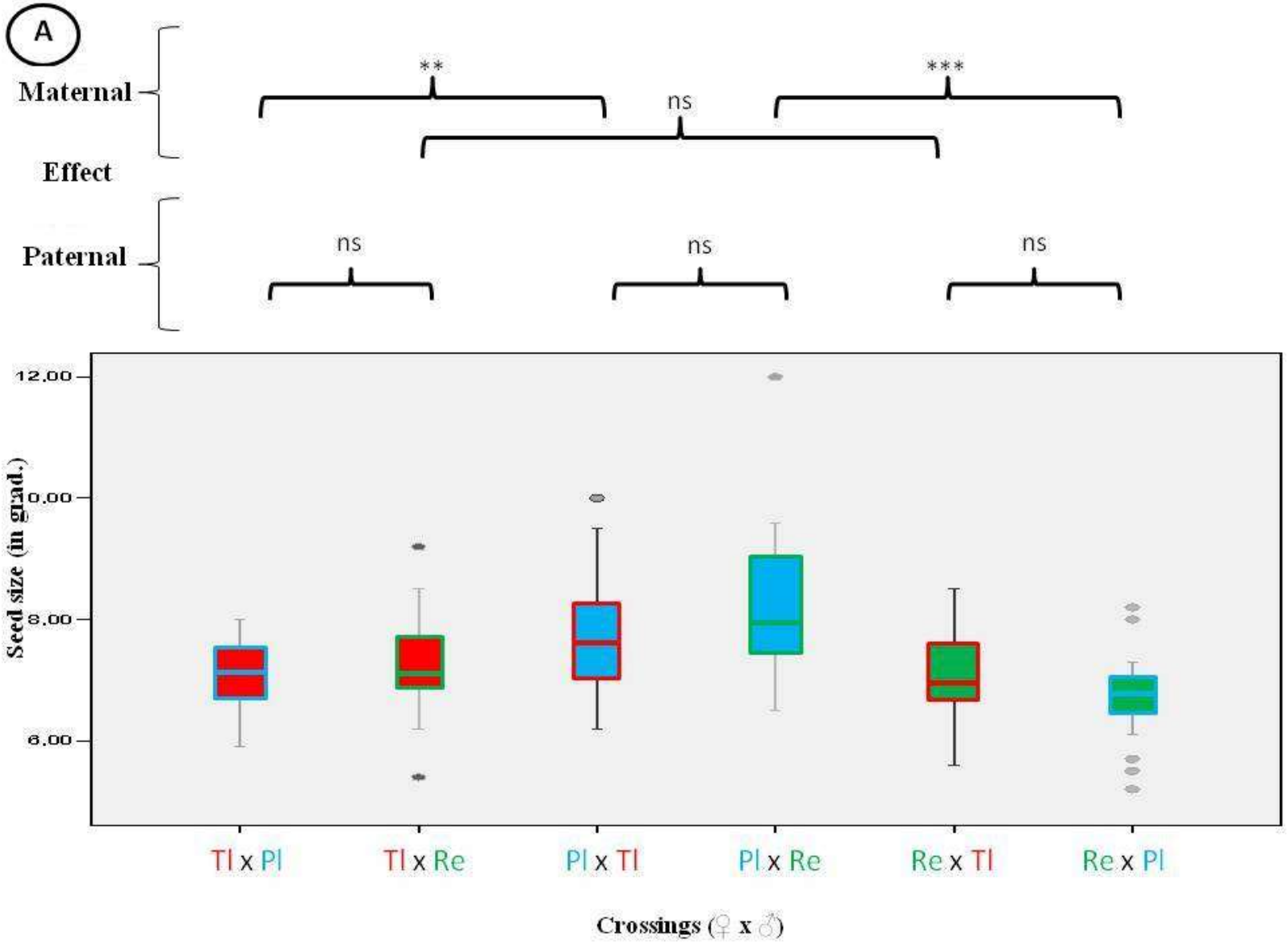

E1
Percentage of androecium-mutant flowers per population
0,500
0,400
0,300
0,200
0,100
0,000
y = -0,0039x + 0,3768
R² = 0,3352
0
20
40
60
80
100
120
Number of polymorphic AFLP-fragment per population

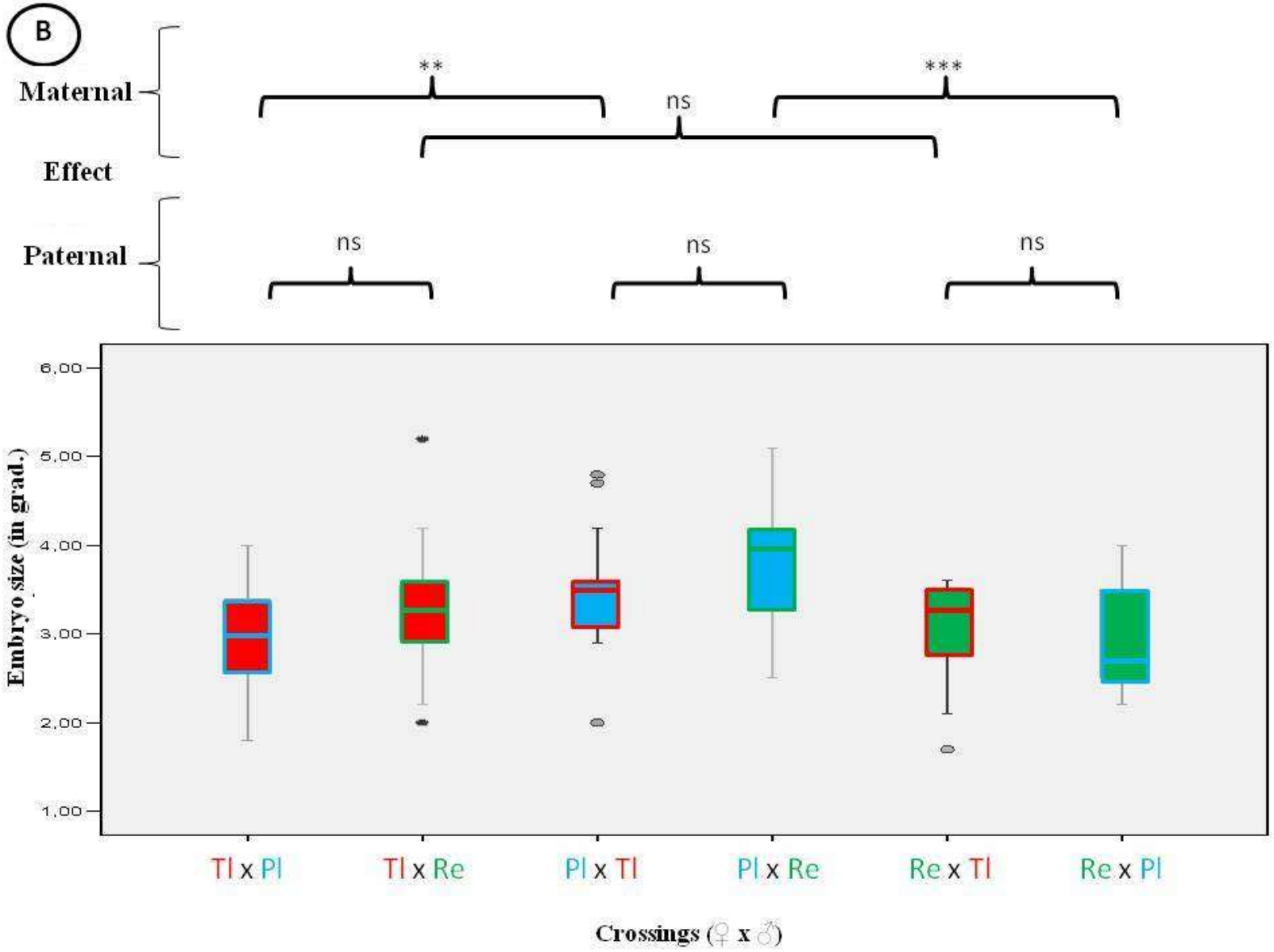

E2
Number of flowers on the number of flowering "putative" individuals per population
6
5
4
3
2
1
0
y = 7,3148x + 1,5847
R² = 0,1493
0
0,05
0,1
0,15
0,2
0,25
0,3
Percentage of polymorphic AFLP-fragment per population

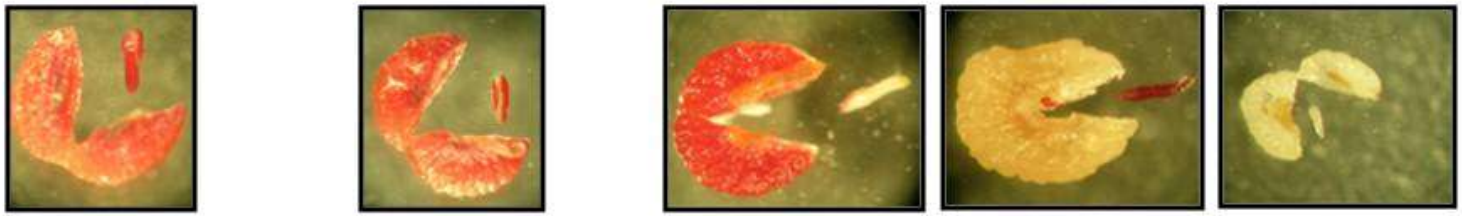

A
1mm
B
1mm
C
1mm
D
1mm


A
Embryo size
Seed size
1,00
2,00
3,00
4,00
5,00
6,00
8,00
10,00
12,00
Tübingen Lustnau
Tl
Pliezhausen
Pl
Reutlingen
Re
**
***
ns
Populations of the triangle

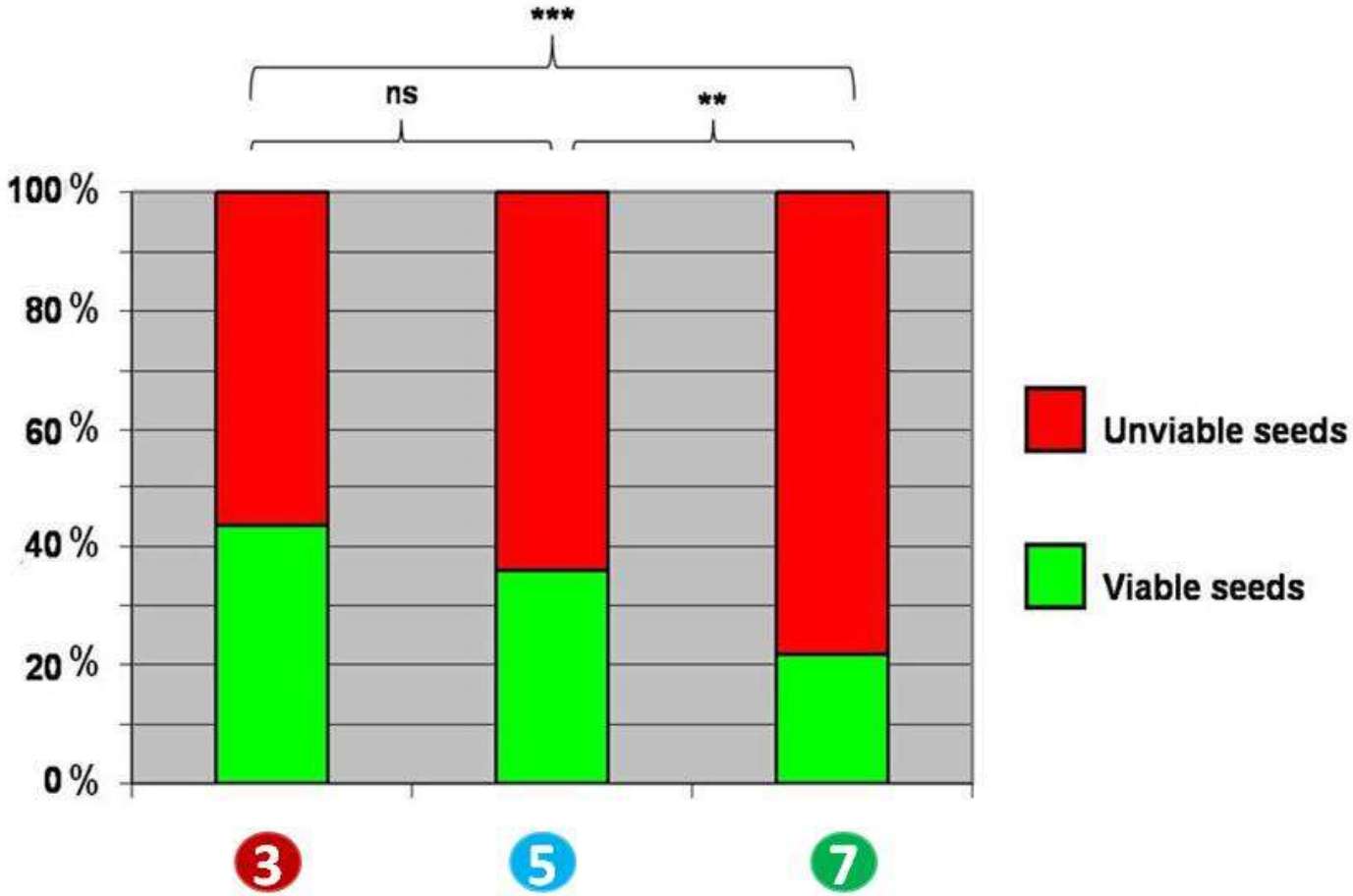

B
Seed size
Embryo size
Lagodekhiregion Lg
Batumiregion Bt
Bakuriani region Bk
ns
ns
ns
ns
*
**


A
Maternal
Effect
Paternal
**
ns
***
ns
ns
ns
12.00
10.00
8.00
6.00
Seed size (in grad.)
Tl x Pl
Tl x Re
Pl x Tl
Pl x Re
Re x Tl
Re x Pl
Crossings (♀ x ♂)


B
Maternal
Effect
Paternal
**
ns
***
ns
ns
ns
Embryo size (in grad.)
6,00
5,00
4,00
3,00
2,00
1,00
Tl x Pl
Tl x Re
Pl x Tl
Pl x Re
Re x Tl
Re x Pl
Crossings (♀ x ♂)

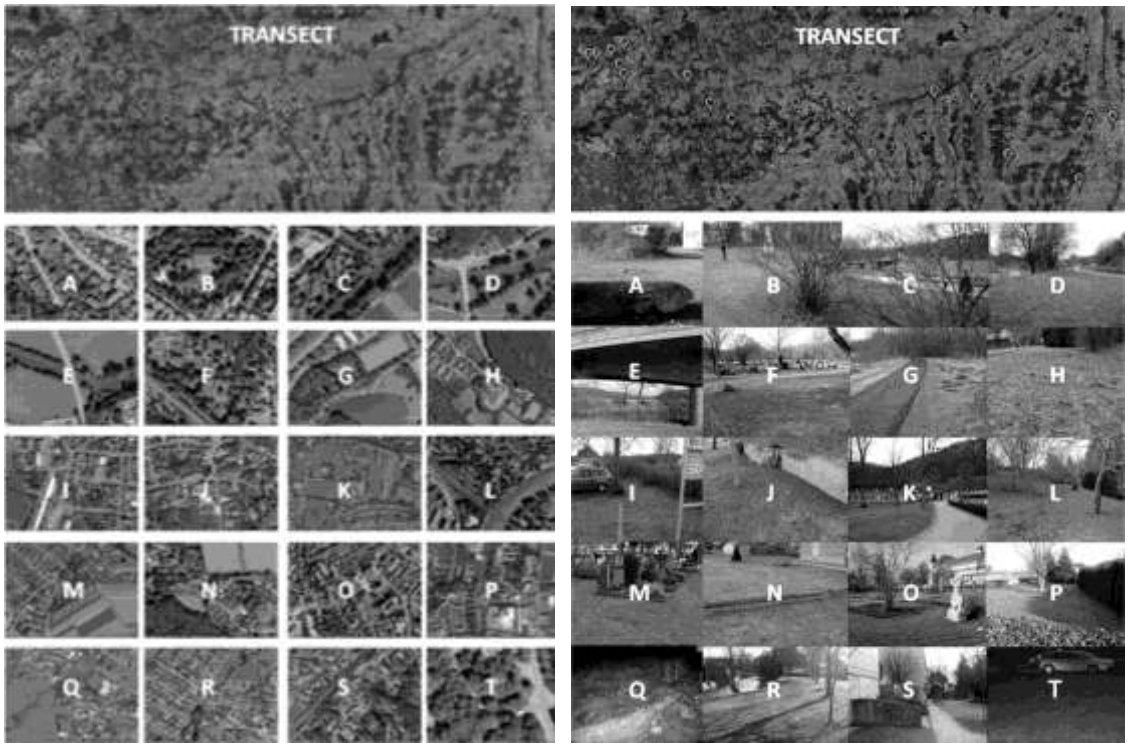


A B C1 C2 C3


***
ns
**
100 %
80 %
60 %
40 %
20 %
0 %
3
5
7
Unviable seeds
Viable seeds

“DIVERSITY OF LOCALITY IN *VERONICA FILIFORMIS*”


P
K
B
R
H
M
M
B
U
W
G
B
W
A
I
Z
G



A	Tübingen (private garden)
B	Tübingen (old botanical garden)
C	Tübingen (lustnau)
D	Pliezhausen
E	Kirchentellinsfurt
F	Betzingen
G	Reutlingen
H	Hohenwittlingen
I	Münsingen
J	Mehrstetten
K	Blaubeuren
L	Ulm
M	Wiblingen
N	Illerzell
O	Günzburg
P	Burgau
Q	Ziemetshausen
R	Großaitingen
S	Westheim bei Augsburg
T	Augsburg

5 SHORT RESEARCH NOTES:

5.1 NOTES ABOUT FLOWER PRODUCTION

***"Induction of flower production in* Veronica"**

by R. Scalone[1], M. Kolf[1] and Dr. rer. nat. K. Bull-Herenu[1]

from:

[1] Institut für Spezielle Botanik und Botanischer Garten, Johannes Gutenberg-Universität Mainz, Germany

5.1.1 Aims

To be able to cross plants, methods to induce flower production were tested. The flowering of *V. persica* and *V. filiformis* has first been observed by Kolf M. and Scalone R. during the greenhouse experiments necessary for papers I and III. Then, an experiment was conducted in collaboration with Dr. rer. nat. Bull-Herenu K. to determine which parameter induces the flowering period of *V. filiformis* (vernalization, day / night cycle, temperature).

5.1.2 Materials and Methods

Observation of the flowering period in *V. persica*

During the study of pollen-ovule ratio for paper I ("Evolution of P-O in *Veronica*" by Scalone *et al.*), a controlled-environment experiment was conducted by Kolf M. to test if the amount of light had an effect on the production of pollen and ovule in the flowers of *V. persica*. Seeds of this species from a single genetic lot provided by a professional seed merchant (see paper I) were sown. Seedlings were randomly assigned to four separate pots, but with the same habitat conditions (greenhouse of the botanical garden). One pot was transplanted into a sandy soil (sandy soil + CC), while the three others were replanted in the same soil (soil composition can be provided upon request by Prof. Dr. Albach). One pot received additional fertilizer (once a week; CC + fertilizer) while another pot received additional light during eight hours every day for the period of the experiment (four months; CC + light). The last pot did not receive additional light and fertilizer and corresponds to our negative control (CC). The number of mature flower buds were noted every day during almost four months while the daily quantity of sunlight was recorded during this period, too (Figure 1). A non-parametric test of Kendall-Tau was conducted to determine whether a significant relationship exists between the number of mature buds (or flowers) and the period of sunshine on day t = 0, -1, -2, -3, -4 and -5 (Table 1) in the negative control of *V. persica*.

Observation of the flowering period in *V. filiformis*

During the elaboration of paper III ("Degradation of sexual reproduction in *V. filiformis* after introduction to Europe" by Scalone & Albach), a transplantation experience of German populations was conducted in *V. filiformis* by Scalone R. to achieve intra- and inter-population crossings. Eighteen populations from Southern Germany (Baden-Württemberg and Bavaria) have been transplanted into one of the glasshouses of the botanical garden of Mainz during the spring of 2007. All populations were transplanted into the same soil and fertilizers were given to maximize the success of transplantation. The number of flowers per flowering individuals (Figure 2) was recorded over nearly two months, as well as the temperature and the period of sunshine (Figure 3). A non-parametric test of the Spearman correlation coefficient was performed to determine whether a significant relationship exists between the number of flowers produced by flowering individuals and the temperature or the period of sunshine on day t = 0, -1, -2, -3, -4 and -5 (Table 2) in *V. filiformis.*

Experiment on the induction of flowering in *V. filiformis*

Five fragments from each of four individuals coming from the population of Assinghausen (Sauerland, individuals noted S1-S2-S4-S8) were transplanted in order to have a total of twenty fragments by pot with the same genetic background. All fragments were at least 4 cm long with one or more pairs of leaves and adventitious roots in order to succeed the transplantation. The soil used for transplantation was the same for each treatment and fertilizers were added as noted previously. Four different treatments were applied to the four genetically-identical pots of *V. filiformis*:

- The first pot was placed in the refrigerator at 5 ° C for one week to simulate the winter period (vernalization) before pot was put in the shadow of a fruit tree in the botanical garden for two weeks (= winter treatment; Figure 4_{A1} & 4_{A2}).
- The second pot was placed directly in the shadow of the same fruit tree for the three weeks of the experiment and corresponds to the negative control (= negative control; Figure 4_{B1} & 4_{B2}).
- The third pot was covered daily by an opaque cover to induce a photoperiod of only eight hours of light per day (from 10a.m. to 6p.m.) in the shadow of the same fruit tree. So, these fragments of *V. filiformis* have day/night cycles of 8h/16h (= short day; Figure 4_{D1} & 4_{D2}).

- The fourth pot had the same treatment as the third one (day/night cycle of 8h/16h) but it was placed during the night period of 16h in the refrigerator at 5 ° C (= cold night; Figure 4_{C1} & 4_{C2}).

Thus, the comparison between the first pot with the second one allows us to see the effect of vernalization on the induction of flowering. The comparison of the second pot with the third one allows us to see the effect of day/night cycle with 8h/16h on the induction (short day). While the comparison of the second and fourth pots allows us to see the effect of temperature during the night period on the induction of flowering. All pots of genetically-identical *V. filiformis* fragments had the same watering and fertilization, the same soil composition and the same environmental conditions (shadow of fruit tree in the botanical garden of Mainz) during the month of the experiment.

5.1.3 Results

The observation of the flowering period in *V. persica* suggests that the production of flowers of this species could be induced by the period of sunshine that occurred three days before the day of flowering ($\tau = 0.098$). However the relationship between the flowering period and the period of sunshine was not significant (p-value = 0.174; Table 1). The observation of the flowering period in *V. filiformis* suggests that the production of flowers of this species appears to be induced by a decrease of the temperature one day ($\rho = - 0.456$) or four days ($\rho = - 0.453$) before the day of flowering. However the relationships between flowering period and air temperature are not significant too, although they were very close to being (p-value = 0.059 & 0.051; Table 2_A). However, a significant negative relationship exists in *V. filiformis* between the flower production at t = 0 and the period of sunshine at the day t = -5, so five days before ($\rho = - 0.485$; p-value = 0.035; Table 2_B). Though, no flower was produced during the experiment testing the effects of the vernalization, day/night cycle (8h - 16h) and cold temperature during the night period on the induction of the flowering in *V. filiformis*, although significant differences of growth can be observed between the four different treatments (Figure 4).

5.1.4 Conclusions

The daily quantity of sunshine plays a repressor role on the flowering period in *Veronica filiformis*. When the period of light per day is short enough, it will induce the production of flowers five days later. The period of five days seems to be necessary, for the plant, to receive the signal, to integrate it and to transmit the information to the floral meristems and to trigger

the flowering period. The quantity of sunshine could also play a repressor role on the flowering period of the sister-species, *Veronica persica*, although a significant effect has not been detected. Also, the temperature seems to play a role on the flowering period, although the relationship is statistically barely non-significant in *V. filiformis* and seems to affect negatively the flowering period. The cold night treatment (night of 16 hours at 5 ° C) seems to be the most appropriate treatment for the fragments of *V. filiformis*. Indeed, the samples under this treatment have improved more their survival and their growth than the samples under three other treatments. The difference of temperature between the day and the night phases seems to induce growth of *V. filiformis* while a short period of sunshine is, probably, necessary to induce the flowering period.

5.1.5 Contents

- **Figure legends**

Fig. 1. Flower number as a function of various parameters (sandy soil, addition of fertilizer or light, negative control) or as a function of the period of sunshine per day

Fig. 2. Flower number per population and per day

Each color corresponds to an area of 30 km of distance. The graphic reveals that populations from the western part of the transect (Baden-Württemberg, e.g. brown pop.) have asynchronous flowering periods compared to populations from the eastern part of the transect (Bavaria, e.g. white pop.).

The populations 4 and 6 (Kirchentellinsfurt and Bretzingen) were collected only in the second year of the experiment (2008), while the population 18 (Großaitingen) never produced flowers. These populations are located on the map but are not represented in the graphic.

Fig. 3. Temperature and period of sunshine per day during the *V.filiformis* transplantation

Fig. 4. Fragments of *V. filiformis* before (from A1 to D1) and after (from A2 to D2) four different treatments

All the fragments come from the same four individuals and grew in the same soil with the same quantities of fertilizers and watering.

The pot A is the winter treatment. The pot B is the negative control. The pot C is the cold night treatment. The pot D is the short day treatment.

- **Table legends**

Table 1. Analysis of the relationships between the number of flower (at day t = 0) and the periods of sunshine in hours for the following days (t = 0 until t= -5) by the non-parametrical Kendall-Tau-test

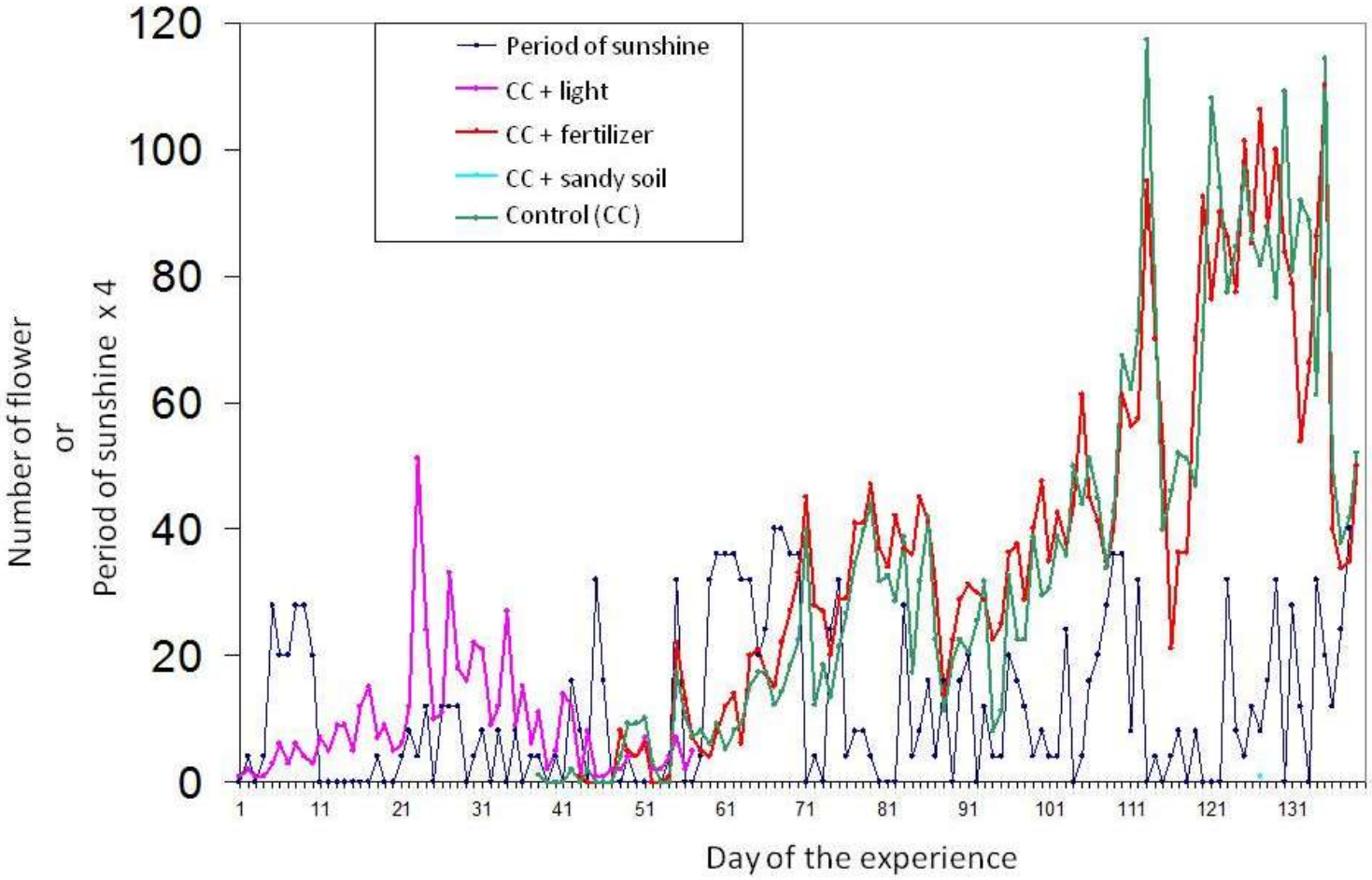
Table 2. Analysis of relationships between the number of flowers produced per flowering individual (FpI) and the temperature (A) or the period of sunshine (B) at t = 0, -1, -2, -3, -4 and -5 by the non-parametrical Spearman´s rank correlation coefficient test

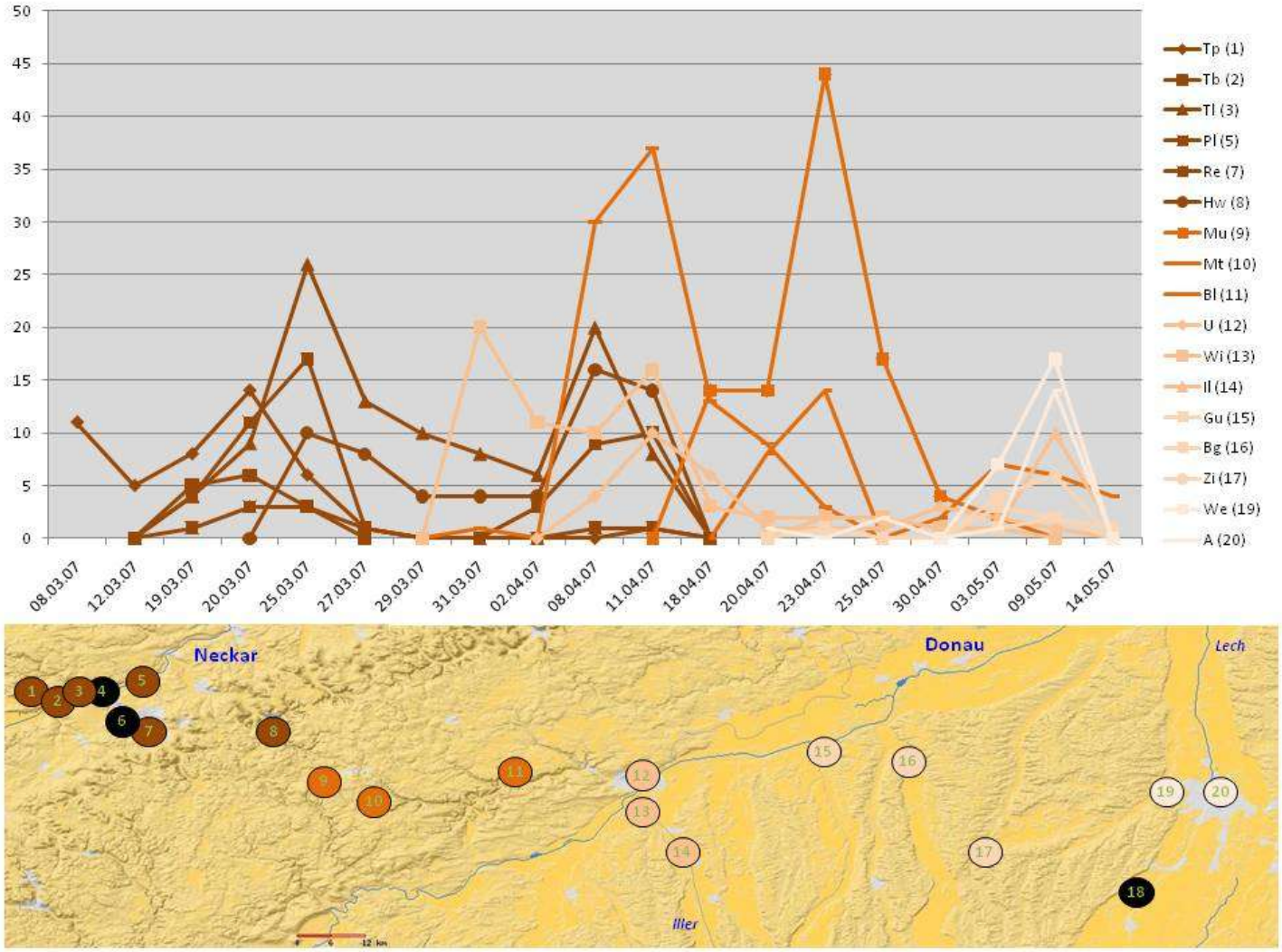
- **Appendix legends**

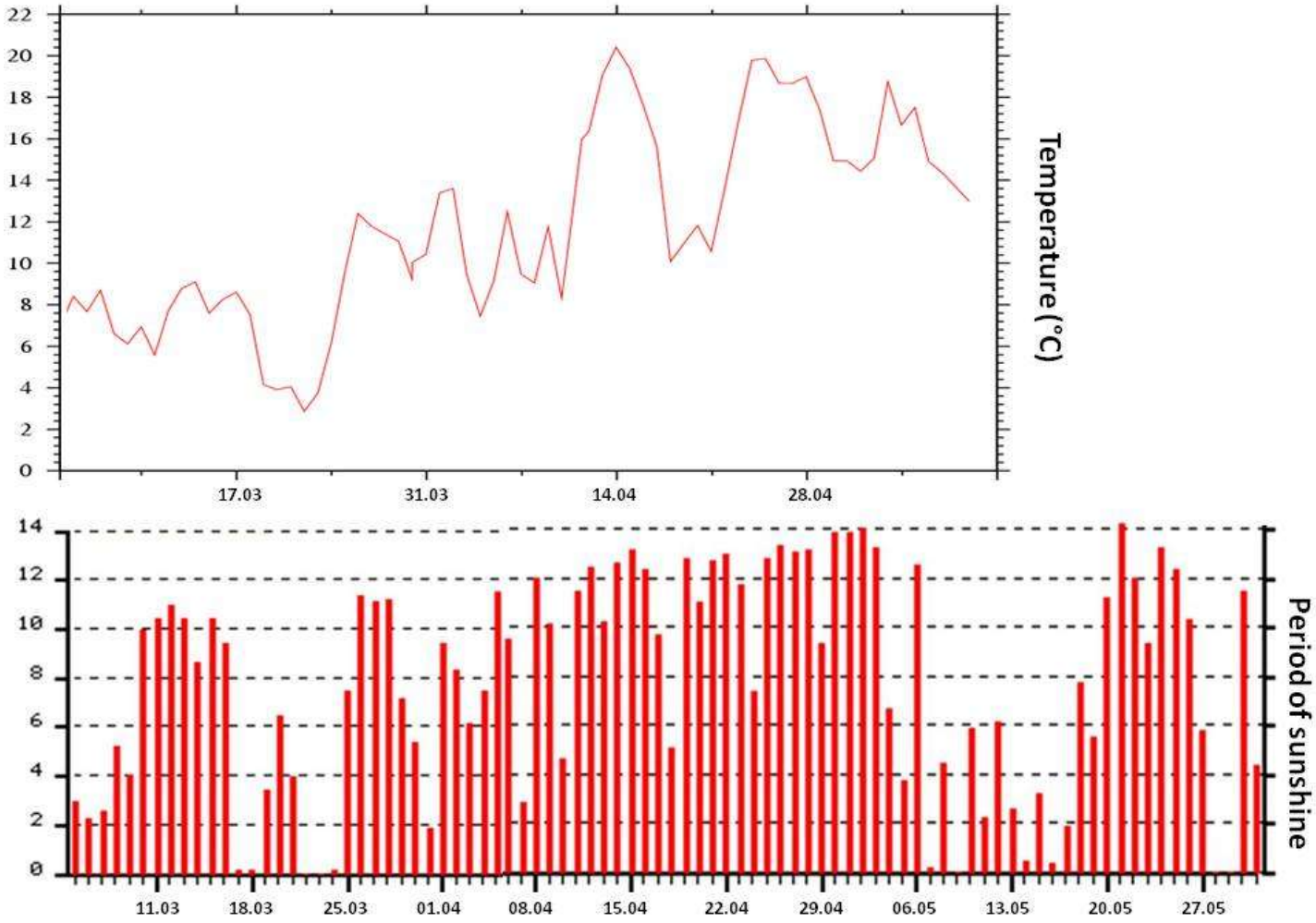
Appendix 1. Experiment on the P-O ratio variation in *V. persica*

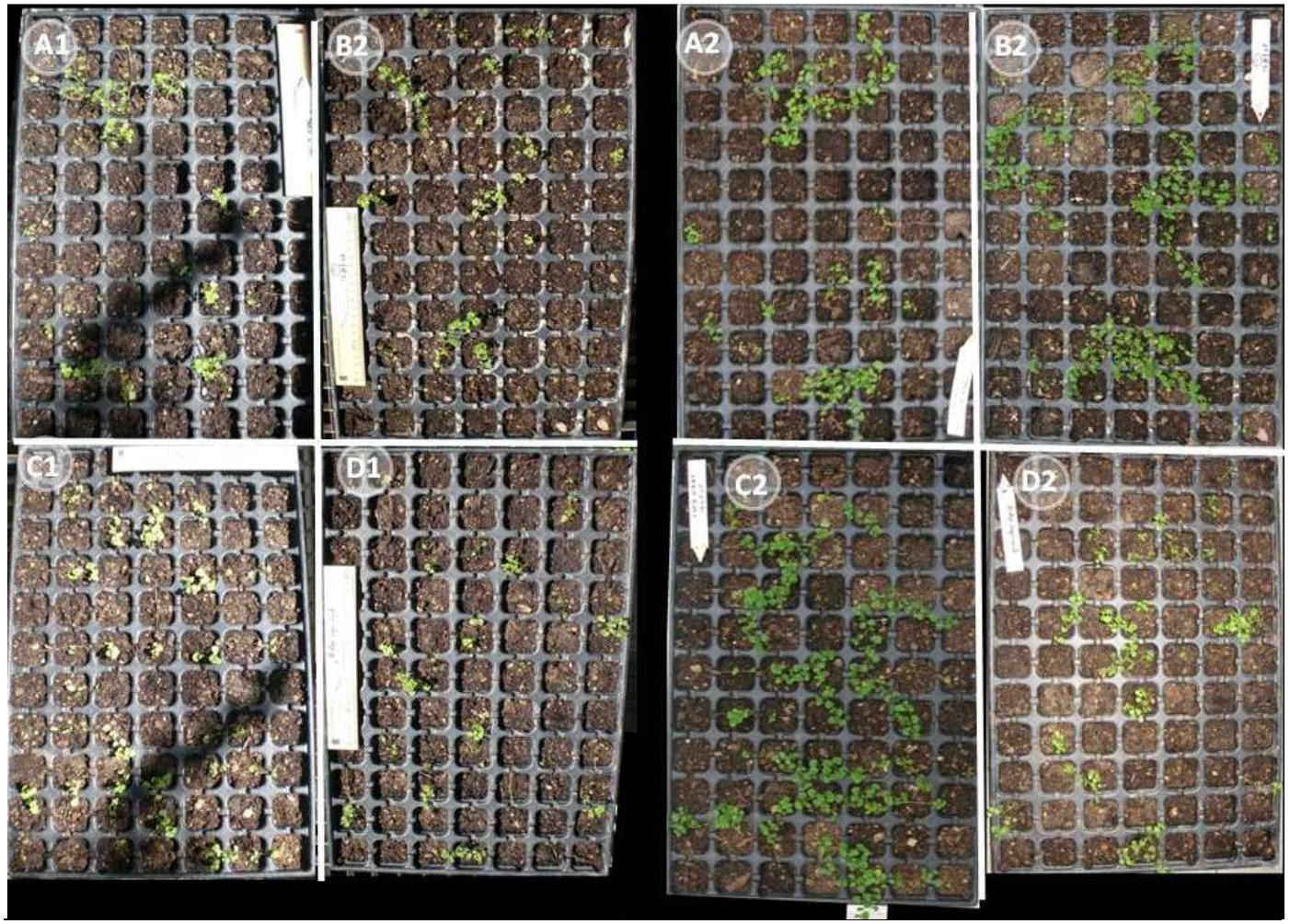
Appemdix 2. Inter-population crossing experiment in *V. filiformis*

Appendix 3. Test on the induction of the flowering period in *V. filiformis*


Period of sunshine
CC + light
CC + fertilizer
CC + sandy soil
Control (CC)
Number of flower
or
Period of sunshine x 4
120
100
80
60
40
20
0
1
11
21
31
41
51
61
71
81
91
101
111
121
131
Day of the experience


50
45
40
35
30
25
20
15
10
5
0
08.03.07
12.03.07
19.03.07
20.03.07
25.03.07
27.03.07
29.03.07
31.03.07
02.04.07
08.04.07
11.04.07
18.04.07
20.04.07
23.04.07
25.04.07
30.04.07
03.05.07
09.05.07
14.05.07
Tp (1)
Tb (2)
Tl (3)
Pl (5)
Re (7)
Hw (8)
Mu (9)
Mt (10)
Bl (11)
U (12)
Wi (13)
Il (14)
Gu (15)
Bg (16)
Zi (17)
We (19)
A (20)
Neckar
Donau
Lech
Iller
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20


22
20
18
16
14
12
10
8
6
4
2
0
17.03
31.03
14.04
28.04
Temperature (°C)
14
12
10
8
6
4
2
0
11.03
18.03
25.03
01.04
08.04
15.04
22.04
29.04
06.05
13.05
20.05
27.05
Period of sunshine


A1
B2
A2
B2
C1
D1
C2
D2

	Period of sunshine	Day	t=0	t=-1	t=-2	t=-3	t=-4	t=-5
Kendall-Tau-test	Coefficient of correlation	Flower	0.017	0.064	0.078	0.098	0.075	0.037
	P-value		0.809	0.372	0.280	0.174	0.304	0.611
	N=	101	101	101	101	101	101	101

A)	Temperature	Day	t=0	t=-1	t=-2	t=-3	t=-4	t=-5
Spearman test	Coefficient of correlation	Fpl	-,356	-,456	-,433	-,370	-,453	-,374
	P-value		,148	,059	,073	,131	,051	,115
	N=		16	16	16	16	17	17

B)	Period of sunshine	Day	t=0	t=-1	t=-2	t=-3	t=-4	t=-5
Spearman-test	Coefficient of correlation	FpI	-0.241	-0.216	-0.276	-0.319	-0.337	-0.485
	P-value		0.319	0.375	0.253	0.184	0.159	0.035
	N=		17	17	17	17	17	17

“DIVERSITY OF SEEDLING FORM IN *VERONICA FILIFORMIS*”

5.2 NOTES ABOUT SELF-STERILITY

***"Evolution of self-sterility in* Veronica"**

by R. Scalone[1]

from:

[1] Institut für Spezielle Botanik und Botanischer Garten, Johannes Gutenberg-Universität Mainz, Germany

5.2.1 Aims

To understand how self-sterility evolves along the time and particularly in *Veronica*, cytological and molecular studies were performed. Investigations on the molecular level with DNA and RNA have been attempted to find the molecule responsible for self-incompatibility within Plantaginacea species (S-RNase). A cytological approach was used in species of *Veronica* to locate the place of action of this molecule responsible for the destruction of self-pollen and preventing, then, the self-fertilization.

5.2.2 Materials and methods

Amplification of the S-RNase from DNA and RNA of Plantaginacean species

Leaves of ten self-sterile species belonging to ten different genera of Plantaginaceae (Figure 1) were collected in the botanical garden of Mainz in order to extract the DNA and try to amplify the S-RNase gene by Polymerase Chain Reaction (PCR) with the primers S2F-S2R[1], S4R-S4F[1], S5F-S5R[1] and Ants2s3F-Ants2s3R[2] ([1] Xue *et al.*, 1996; [2] Vieira & Charlesworth, 2002). Styles from flowers of these species were also collected in liquid nitrogen to extract total RNA and amplify the transcript of the S-RNase by Reverse Transcription-PCR (RT-PCR) and clone it. This methodology was successfully used by Vieira & Charlesworth (2002) in the genera *Antirrhinum* and *Misopates*. So, the species *Antirrhinum molle* present in the botanical garden of Mainz was used as positive control for this experiment. Protocols for extracting DNA and RNA as well as those of PCR, RT-PCR and cloning followed those published by Xue *et al.* (1996) and Vieira & Charlesworth (2002). The sequences derived from DNA or RNA extractions of *Plantaginaceae* or *Veronica* were blasted in NCBI site (http://blast.ncbi.nlm.nih.gov/) to determine whether these sequences correspond to the gene of the S-RNase.

Pollen tube growth experiments

A minimum of 5 different individuals for each of the three *Veronica* species tested (*V. filiformis*, *V. gentianoides* and *V. teucrium*) were covered to conduct hand-controlled pollinations (between 10 and 20 flowers; Appendix 1). Emasculations, self- and cross-pollinations were made 24 hours before pistil collection. Pollinated (five per self- and cross-pollinations) and virgin (one negative control) styles were fixed for 24 hours in AFE (ethanol, formaldehyde, acetic acid, 8:1:1). The AFE-fixative was removed by pipetting and washed five times with water. Afterwards, pistils were incubated in 5N NaOH until they were transparent. More than 24 hours later, the pistils were washed 15 times in water. Finally, the complex carbohydrate "callose" (β-1, 3, glucan) produced by mature pollen tubes was stained by decolored aniline blue overnight in a dark room. Afterwards pistils were cleaned five times in water, put in glycerine on a slide and slightly squashed under a covering slip. Preparations were observed with UV- or blue-excitation under a fluorescence microscope (Leitz Diapland & Leitz Wetzlar). The hand-pollinated flowers, which were not collected for the pollen tube growth treatment, remained covered until the end of the flowering period in order to check their fruit production after self- or cross-pollinations.

5.2.3 Results

Only within the positive control of *Antirrhinum molle* a sequence corresponding to the gene for the S-RNase was found. This sequence of 463 base pairs is derived from the amplification of total RNA by RT-PCR with the primers Ants2s3F-Ants2s3R after cloning (Appendix 2_A). This nucleic sequence encodes for a protein composed by 154 amino acids (aa, Appendix 2_B), which has five amino acids (aa) in common to the conserved domain C1of the S-ribonuclease sequence from *Antirrhinum hispanicum* subsp. *mollissimum* (WPFS-C), eight aa to the domain C2 (FTIHGLWP), five aa to the domain C3 (KHG-CS) and five aa to the domain C4 (LL-RFN) (Appendice 3 & 4). Our new protein presents also common amino acids with the previously cited S-RNase located in the two hypervariable domains HV (HVa: 7 / 16 aa, HVb: 7 / 12 aa; Appendix 4). This new protein has a best score of 185 homology (E-value: 2×10^{-57}; Appendix 4) with the sequence of *A. hipanicum* registered in Genbank http://www.ncbi.nih.gov/) under the code CAD29435. Thus, this new sequence is phylogenetically closer to the S-RNase of *A. hispanicum* subp. *mollissimum* than to the other alleles of S-RNase present in *A. molle* (SA, SC & S6 alleles; Figure 2).

Three *Veronica* species from three different subgenera were tested by crossing and pollen tube growth experiment (Figure 3). The morphology of *Veronica* virgin pistil is relatively

simple and structured by a stigma with papillary cells at the top, a long style including two xylem tubes and an ovary with two locules (e.g., Figure 3_A). The positive controls (outcrossing) show the pollen grains (Figure 3_{D1}) producing pollen tubes growing into the style (yellow signal in Figure 1_{D2}) and further to the ovules in the ovary (24 hours after pollination). At the entrance of the ovary, the pollen tubes separate in the directions of the two locules (Figure 3_{D3} – positive control of *V. gentianoides*). These descriptions are common to the three different *Veronica* species tested (*V. filiformis* – Figure 3_B; *V. gentianoides* – Figure 3_D; *V. teucrium* – Figure 3_F). Self-pollinations show pollen grains (Figure 3_{E1}) producing pollen tubes growing into the style (yellow signal in Figure 3_{E2}) too. However, the pollen tubes do not arrive at the ovules (24 hours after pollination), but are stopped before reaching the ovary in *V. filiformis* (Figure 3_C) and *V. gentianoides* (Figure 3_E). This is not the case for *V. teucrium* (Figure 3_G), where self-pollen tubes enter the ovary. This last species was also the only species to produce capsules after self-pollination (Appendix 5).

5.2.4 Conclusions

The finding of a new allele of S-RNase present in specimens of the *A. molle* species located in the botanical garden of Mainz proves that botanical gardens could still be a source of undiscovered diversity in the case of the genetic system of S -locus (S-RNase & SLF genes). Although the protocol is extremely expensive (RNA extraction, RT-PCR, cloning, sequencing) and no species of *Plantaginaceae* gave significant results, except for the genus *Antirrhinum*, the free access to this diversity should help research groups located closely to botanical gardens to create projects and investigations on the molecular evolution of self-incompatibility, principally within the thirty species of *Antirrhinum* and twenty species of the sister-genus *Linaria*. The conservation of the floral form within the genus *Veronica* seems to be accompanied by a conservation of the genetic self-incompatibility system. Indeed, our crossings and pollen tube growth experiments gave evidence for the presence of a gametophytic type of self-incompatibility in *V. gentianoides* (*V.* subg. *Beccabunga*; Figure 1) as previously suggested by Correns (1920) and in *V. filiformis* (*V.* subg. *Pocilla*; Figure 1) confirming the results of Lehmann (1944). The results of these experiments also corroborated the facultative xenogamous character of *V. teucrium* (*V.* subg. *Pentasepala*; Figure 1) as suggested by Scheerer (1937). The presence of a gametophytic self-incompatibility (GSI) system in at least two different subgenera of *Veronica* allows affirming that this GSI genetic system is conserved in *Veronica* and could be homologous to the GSI-system of *Antirrhinum* using S-RNase and SLF molecules (Vieira & Charlesworth 2002).

5.2.5 Acknowledgements

We thank Prof. R. Classen-Bockhoff for access to her fluorescence microscope, Dr. A. Ley for helping with the pollen tube growth protocol, Ms. D. Franke for the figures of pollen tube growth experiment. This study was supported by the DFG (AL632 / L-1) and the FWF (P18598).

5.2.6 References

Albach D. C. and Chase M. W. (2004) Incongruence in *Veroniceae* (Plantaginaceae): Evidence from two plastid and a nuclear ribosomal DNA region. *Molecular Phylogenetics and Evolution* **32:** 183–197.

Albach D. C. and Meudt H. M. (2010) Phylogeny of *Veronica* in the Southern and Northern Hemispheres based on plastid, nuclear ribosomal and nuclear low-copy DNA. *Molecular Phylogenetics and Evolution* **54:** 457-471.

Correns C. (1920) Vererbungsversuche mit buntblättrigen Sippen. III. *Veronica gentianoides albocincta*. *Sitzungsberichte der Preussischen Akademie der Wissenschaften zu Berlin* 212-217.

Lehmann E. (1944) *Veronica filiformis* Sm., eine selbsterile Pflanze. *Jahrbücher für wissenschaftliche. Botanik* **91:** 395-403.

Scheerer H. (1937) Experimentelle und zytologische Untersuchungen innerhalb der *Veronica*-Gruppe *Pentasepala*. *Flora* **131:** 287.

Vieira C. P. and Charlesworth D. (2002) Molecular variation at the self-incompatibility locus in natural populations of the genera *Antirrhinum* and *Misopates*. *Heredity* **88**: 172-181.

Xue Y., Carpenter R., Dickinson H., Coen E. (1996) Origin of allelic diversity in *Antirrhinum* S-locus RNases. *The Plant Cell* **8:** 805-814.

5.2.7 Contents

- **Figure legends**

Fig. 1. Presence of the self-sterility along the phylogeny of Plantaginaceae (left) and *Veronica* (right)

The genera of Plantaginaceae labelled in red are the ones including self-sterile species which were tested during the molecular experiments. The *Veronica* phylogeny represented here (blue box) is based on previous works (Albach *et al.*, 2004; Albach & Meudt, 2010) and made by Bayestraits-analysis. The species of *Veronica* labelled in green are self-compatible, while

the red ones are self-sterile (the three underlined ones were tested by pollen tube growth experiments). Note that data for *Veronica cusickii* was inserted at the phylogenetic position of *V. copelandii*, data for *V. dillenii* at the position of *V. verna*, data for *V. incana* at the place of *V. barrerlieri* and data for *V. vendetta-deae* at the place of *V. baranetzkii*.

Fig. 2. Phylogeny of S-RNase sequences in *Antirrhinum* genus (Plantaginaceae)

The new sequence found by RNA-extraction, RT-PCR and cloning in *A.molle* is in the red box.

Fig. 3. Pollen tube growth experiments in three putative xenogamous species: *V. filiformis* (3_A, 3_B, 3_C), *V. gentianoides* (3_D, 3_E) and *V. teucrium* (3_F, 3_G)

Figure 3_A is a virgin carpel and corresponds to the negative control. Figures 3_B, 3_D and 3_F are the cross-pollinated carpels of *V. filiformis*, *V. gentianoides* and *V. teucrium* and correspond to the positive controls. Figures 3_C, 3_E and 3_G are the self-pollinated carpels of *V. filiformis*, *V. gentianoides* and *V. teucrium* respectively. The figures 3_{D1}, 3_{D2}, 3_{D3} and 3_{E1}, 3_{E2}, 3_{E3} are enlargements of the pistil, the style and the entrance of ovaries for the cross- and self-pollinated carpels of *V. gentianoides*.

- **Appendix legends**

Appendix 1. Bagged individual of *V. teucrium* after self-pollination and emasculation

Appendix 2. Nucleic (A) and protein (B) sequences of the new allele of the S-RNase from *Antirrhinum molle*

Appendix 3. BLAST of the new nucleic sequence of *Antirrhinum molle* with the nucleic sequence of the S-ribonuclease of *Antirrhinum hispanicum* subsp. *mollissinum*

The nucleic sequences marked in green are similar between *A. molle* and *A. hispanicum* subsp. *mollissinum* for the S-RNase while the red ones are different.

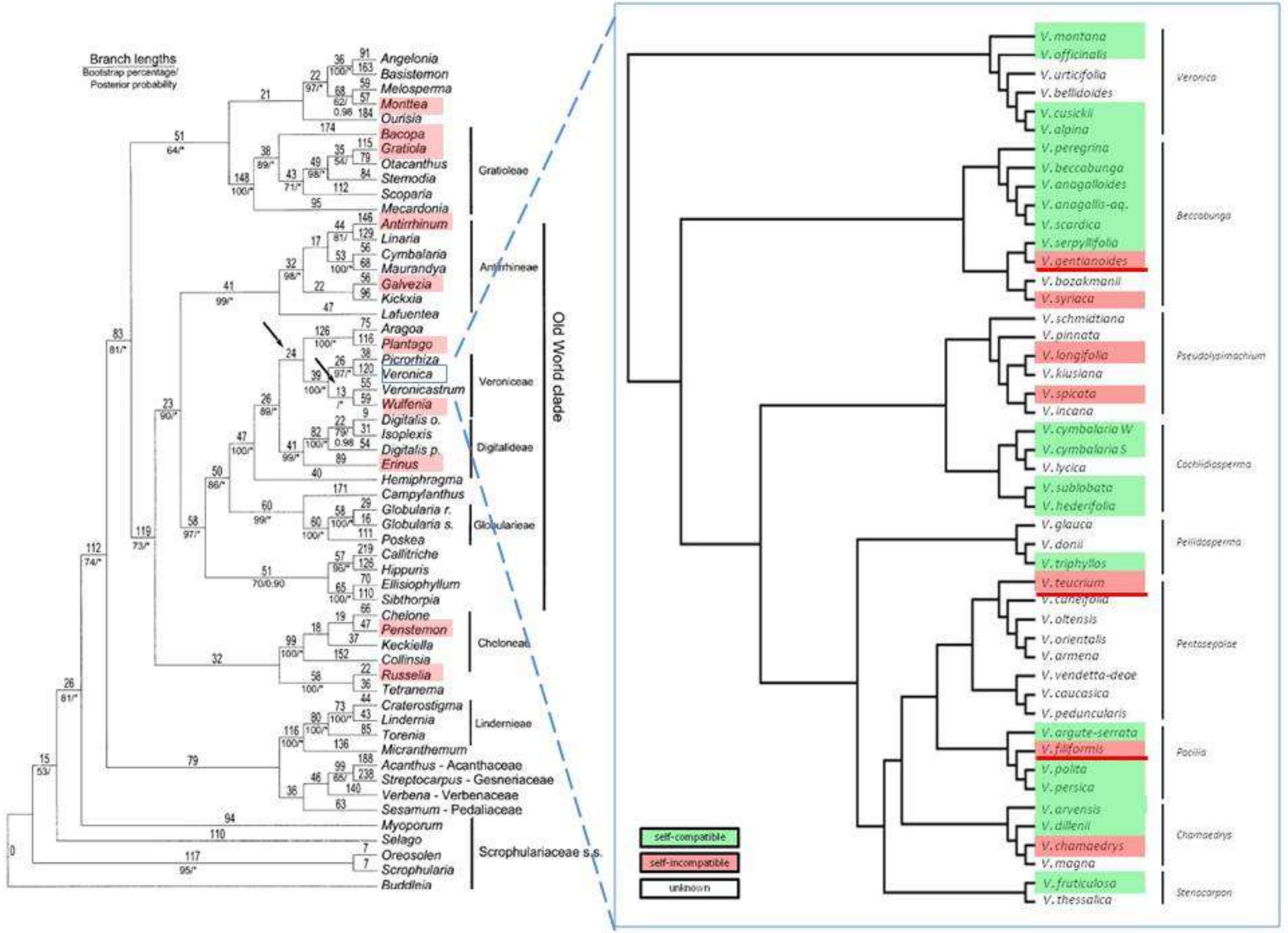
Appendix 4. BLAST of the new protein sequence of *Antirrhinum molle* with the protein sequence of the S-ribonuclease of *Antirrhinum hispanicum* subsp. *mollissinum*

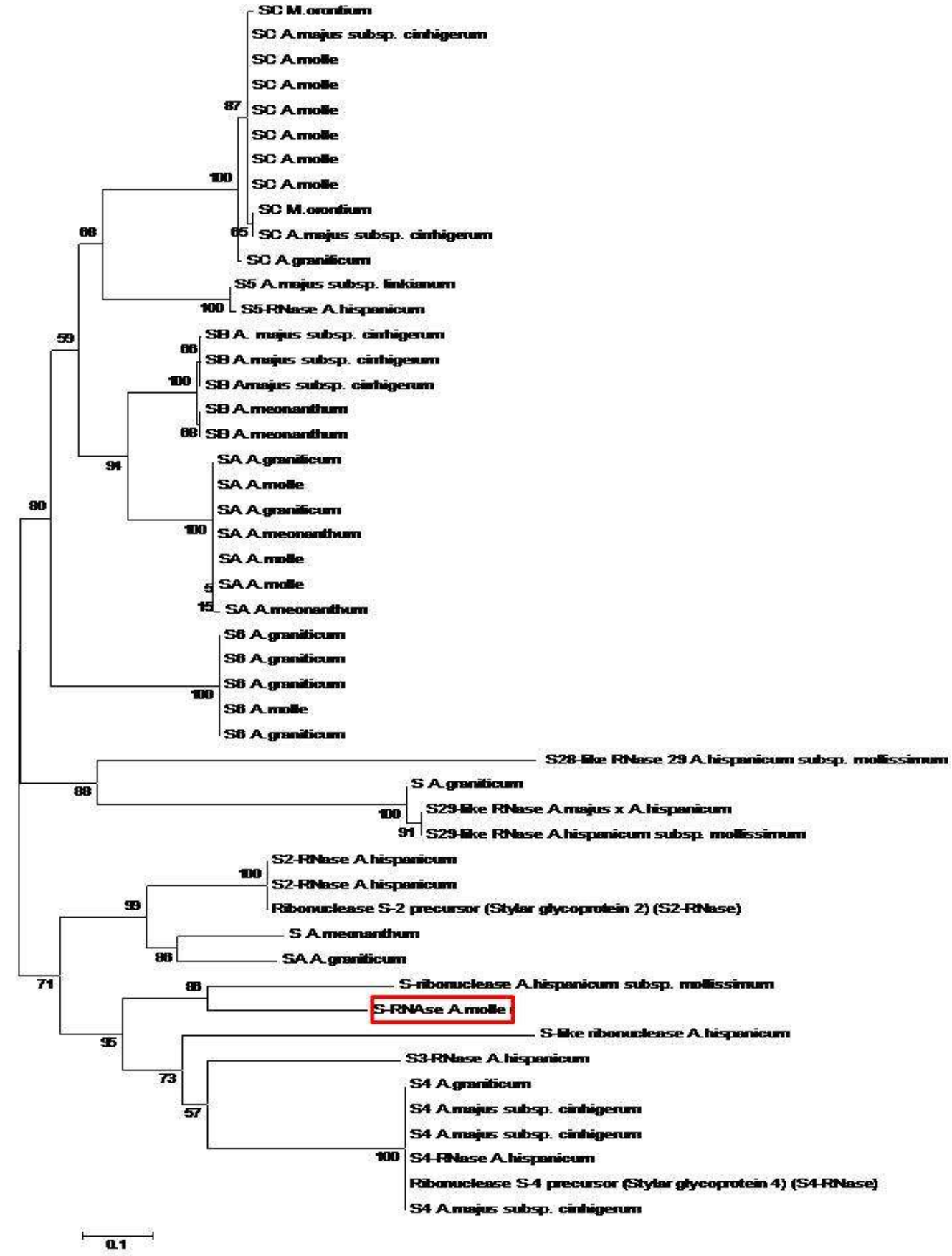
The protein sequences marked in green are similar between *A. molle* and *A. hispanicum* subsp. *mollissinum* for the S-RNase while the red ones are different. The orange sequences are different but present the same functions within the protein. The underlined sequences are the

conserved (C1, C2, C3) or the hypervariable (HVa, HVb) domains of the protein, which have important roles in the “lock-and-key” function of the S-RNase.

Appendix 5. Capsule production after self-pollination in *V. teucrium*

The black arrows indicate the position of capsules on the stem.


Branch lengths
Bootstrap percentage/
Posterior probability
Angelonia
Basistemon
Melosperma
Monttea
Ourisia
Bacopa
Gratiola
Otacanthus
Stemodia
Scoparia
Mecardonia
Antirrhinum
Linaria
Cymbalaria
Maurandya
Galvezia
Kickxia
Lafuentea
Aragoa
Plantago
Picrorhiza
Veronica
Veronicastrum
Wulfenia
Digitalis o.
Isoplexis
Digitalis p.
Erinus
Hemiphragma
Campylanthus
Globularia r.
Globularia s.
Poskea
Callitriche
Hippuris
Ellisiophyllum
Sibthorpia
Chelone
Penstemon
Keckiella
Collinsia
Russelia
Tetranema
Craterostigma
Lindernia
Torenia
Micranthemum
Acanthus - Acanthaceae
Streptocarpus - Gesneriaceae
Verbena - Verbenaceae
Sesamum - Pedaliaceae
Myoporum
Selago
Oreosolen
Scrophularia
Buddleia
Gratioleae
Antirrhineae
Veroniceae
Digitalideae
Globularieae
Cheloneae
Lindernieae
Scrophulariaceae s.s.
Old World clade
V. montana
V. officinalis
V. urticifolia
V. bellidoides
V. cusickii
V. alpina
V. peregrina
V. beccabunga
V. anagalloides
V. anagallis-aq.
V. scardica
V. serpyllifolia
V. gentianoides
V. bozakmanii
V. syriaca
V. schmidtiana
V. pinnata
V. longifolia
V. kiusiana
V. spicata
V. incana
V. cymbalaria W
V. cymbalaria S
V. lycica
V. sublobata
V. hederifolia
V. glauca
V. donii
V. triphyllos
V. teucrium
V. canescens
V. altensis
V. orientalis
V. armena
V. vendetta-dece
V. caucasica
V. peduncularis
V. argute-serrata
V. filiformis
V. polita
V. persica
V. arvensis
V. dillenii
V. chamaedrys
V. magna
V. fruticulosa
V. thessalica
Veronica
Beccabunga
Pseudolysimachium
Cochlidiosperma
Pellidosperma
Pentasepalae
Pocilla
Chamaedrys
Stenocarpon
self-compatible
self-incompatible
unknown


SC M.orontium
SC A.majus subsp. cinhigerum
SC A.molle
SC A.molle
87
SC A.molle
SC A.molle
SC A.molle
100
SC A.molle
SC M.orontium
68
65
SC A.majus subsp. cinhigerum
SC A.granilicum
S5 A.majus subsp. linkianum
100
S5-RNase A.hispanicum
59
SB A. majus subsp. cinhigerum
66
SB A.majus subsp. cinhigerum
100
SB Amajus subsp. cinhigerum
SB A.meonanthum
68
SB A.meonanthum
94
SA A.granilicum
SA A.molle
80
SA A.granilicum
100
SA A.meonanthum
SA A.molle
5
SA A.molle
15
SA A.meonanthum
S6 A.granilicum
S6 A.granilicum
S6 A.granilicum
100
S6 A.molle
S6 A.granilicum
S28-like RNase 29 A.hispanicum subsp. mollissimum
88
S A.granilicum
100
S29-like RNase A.majus x A.hispanicum
91
S29-like RNase A.hispanicum subsp. mollissimum
S2-RNase A.hispanicum
100
S2-RNase A.hispanicum
99
Ribonuclease S-2 precursor (Stylar glycoprotein 2) (S2-RNase)
S A.meonanthum
86
SA A.granilicum
71
88
S-ribonuclease A.hispanicum subsp. mollissimum
S-RNAse A.molle
S-like ribonuclease A.hispanicum
95
S3-RNase A.hispanicum
73
S4 A.granilicum
57
S4 A.majus subsp. cinhigerum
S4 A.majus subsp. cinhigerum
100
S4-RNase A.hispanicum
Ribonuclease S-4 precursor (Stylar glycoprotein 4) (S4-RNase)
S4 A.majus subsp. cinhigerum
0.1


A
D1
D2
D3
E1
E2
E3
B
D
F
C
E
G

A)

Primer Ants2s3F = [AAATTGGTTCTTCCAATGG] ...

…CCATTTTCTGTCTGCAGCCAACCTGAAGCATACTGCCAGAGAGATCCTCTCCCCTTCAG
ATTCACCATCCATGGCCTCTGGCCAGATAATCGTTCCCAGCCGTTGAGCGACTGCGATAC
TGTTCCACTCAACGATATCGTTAACTCAAGATTACTACAGAGGATGAAGCGAAGCTGGCC
AGATCTTTATCAACCAAGAGACATGGGTCCGTCCTACAATTTCTGGTCAGAGCAATGGAA
GAAACATGGGACATGTTCTTTGCCTTTTTACAGCCAGACCAGCTACTTGATTAAAGCATTG
GATTTAACAGAAAGGTTTAACTTACGGAAAATCCTTAGGGAGAAGCGAGTAACTCCTGG
AAGATCTTTCAAACTATCCCGAGTAACAGGAATTATAAAGGCTGAAACTCAAGGCAGCCC
CATAT…

… [TGAAATGCCGCGTGAAGGTT] = Primer Ants2s3R

B)

Primer Ants2s3F = [NWFFQW] …

…PFSVCSQPEAYCQRDPLPFRFTIHGLWPDNRSQPLSDCDTVPLNDIVNSRLLQRMKR
SWPDLYQPRDMGPSYNFWSEQWKKHGTCSLPFYSQTSYLIKALDLTERFNLRKILREKRVTP
GRSFKLSRVTGIIKAETQGSPIL…

… [KCRVKV] = Primer Ants2s3R

Score = 244 bits (270)

Expect = 3e-61

Identities = 281/376 (75%)

Gaps = 4/376 (1%)

Strand=Plus/Plus

```
A.molle   7   GTTCTTCCAATGGCCATTTTCTGTCTGCAGCCAACCTGAAGCATACTGCCAGAGAGATCC   66
A.hisp    82  GTTCT-CCATTGGCCATTTTCCTATTGCAGCCTGCCTGATTCGAAGTGCAAAAGAACTCC   140

A.molle   67  TCTCCCCTTCAGATTCACCATCCATGGCCTCTGGCCAGATAATCGTTCCCAGCCGTTGAG   126
A.hisp    141 TCTTCCTTCCATATTTACTATCCATGGTCTATGGCCGAATAACTACAGCCAACCGTTGAG   200

A.molle   127 CGACTGCGATACT---GTTCCACTCAACGATATCGTTAACTCAAGATTACTACAGAGGAT   183
A.hisp    201 CGACTGCAATATTCTGGTTCCCTTCACAAATATATATGACCAACCCTTATTACAAAGGAT   260

A.molle   184 GAAGCGAAGCTGGCCAGATCTTTATCAACCAAGAGACATGGGTCCGTCCTACAATTTCTG   243
A.hisp    261 GATGCGAAGCTGGCCAGATCTTAATCAACCAATGAATACGGGGCCGTCTCACAACTTCTG   320

A.molle   244 GTCAGAGCAATGGAAGAAACATGGGACATGTTCTTTGCCTTTTTACAGCCAGACCAGCTA   303
A.hisp    321 GTCATATCAATGGAAGAAACATGGATCTTGTTCTTTGCCTCGGTACAGCCAGACCAGCTA   380

A.molle   304 CTTGATTAAAGCATTGGATTTAACAGAAAGGTTTAACTTACGGAAAATCCTTAGGGAGAA   363
A.hisp    381 CTTGTTCAAAGCATTGGAGTTATACGACAGGTTTAATGTTCTGCAAATCCTGACGGACGG   440

A.molle   364 GCGAGTAACTCCTGGA                                              379
A.hisp    441 TCGATTGGCTCCTGGA                                              456
```

Score = 185 bits (469)

Expect = 2e-57

Identities = 87/149 (58%)

Positives = 109/149 (73%)

Gaps = 1/149 (1%)

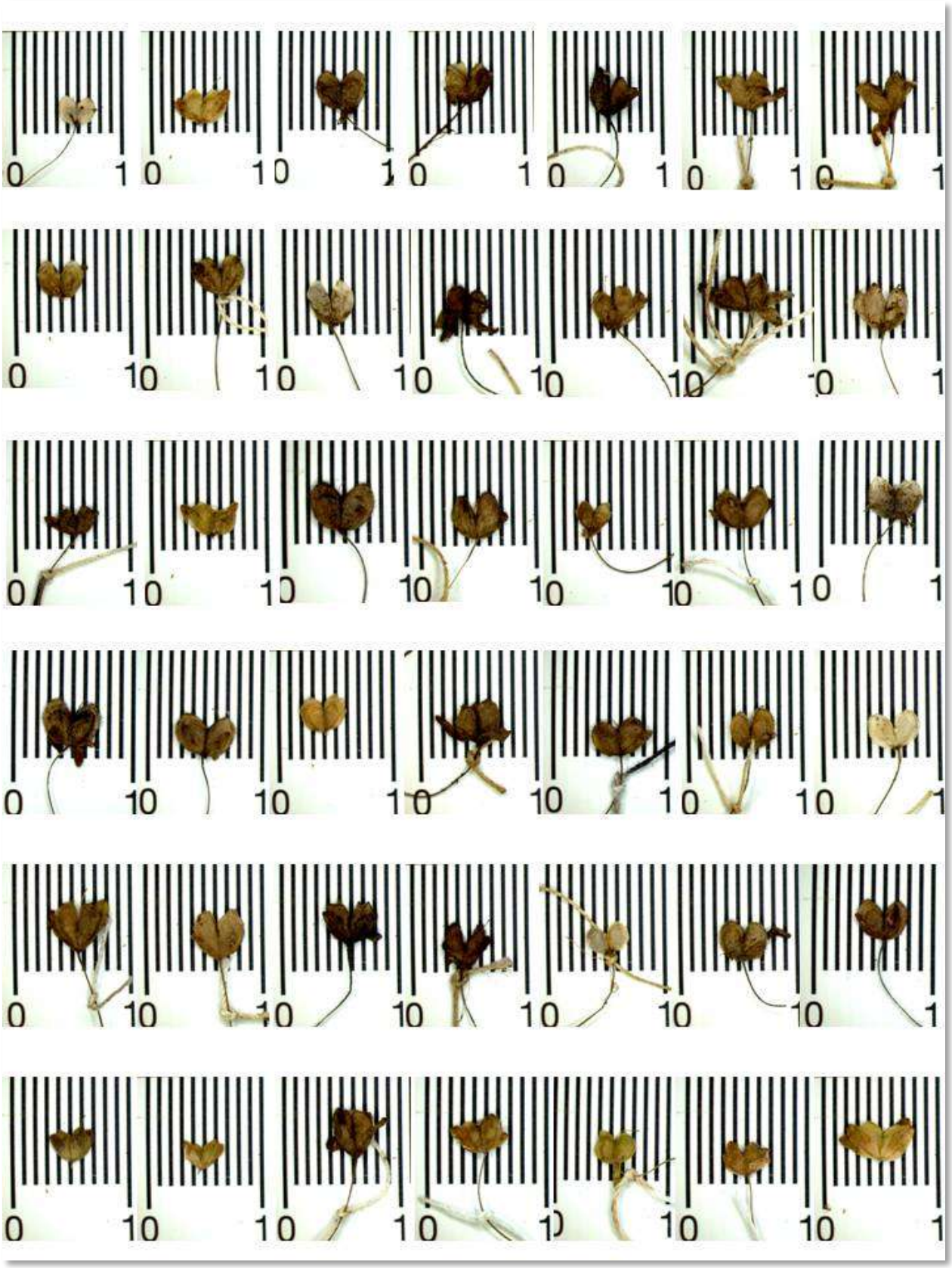
Method: Compositional matrix adjust

```
A.molle   4  FQWPFSVCSQPEAYCQRDPLPFR FTIHGLWPDNRSQPLSDCDT-VPLNDIVNSRLLQRMK  62
A.hisp   29  LHWPFSYCS LPDSKCK RTPLPS I FTIHGLWPNNYSQPLSDCNILVPFT N IYDQPLLQRMM  88
                    C1                          C2                      HVa

A.molle  63  RSWPDLY QPRDMGPSYNFWSEQWKK HGTCSLPFYSQTSYLI KALDLTERFN LRK ILRE KR  122
A.hisp   89  RSWPDLNQPMNTGPSHNFWSYQWKKHGSCSLPRYSQTSYLFKALELYDRFNVLQILTDGR  148
                              HVb          C3              C4

A.molle 123  VTPGRS FK L SRVTGIIKAETQGSPILKCR  151
A.hisp  149  LAPGDNYTVSQINITIIQE I GA I PTVKCR  177
```

“DIVERSITY OF CAPSULE SIZE IN *VERONICA FILIFORMIS*”



5.3 NOTES ABOUT FLOWER PRODUCTION

***"Variation of capsule size in* Veronica filiformis"**

by R. Scalone[1]

from:

[1] Institut für Spezielle Botanik und Botanischer Garten, Johannes Gutenberg-Universität Mainz, Germany

5.3.1 Aims

In the course of crossing experiments conducted during the elaboration of paper III ("Degradation of sexual reproduction in *V. filiformis* after introduction to Europe" by Scalone & Albach), variations in the size of capsules formed were observed. To determine whether these observations are significant, measurements and tests on capsule morphology were performed. To understand the reason for this variation, the relationship of the size of the capsule with temperature and the quantity of sunshine were investigated. Relations with seed viability tested during the paper III were also investigated.

5.3.2 Materials and methods

Variation of the capsule size in *V. filiformis*

During the elaboration of paper III, a crossing experiment with German populations of *V. filiformis* was conducted. Heterogeneity in the size of the capsules produced by crossing was observed. Several measurements of the size of these capsules were conducted (length, width, perimeter, area, ovary length) and tested by Kolmogorov-Smirnov and Shapiro-Wilk tests to see if their distributions were normal or not. The number of seeds and the sizes of these (length and width) for the two putative different types of capsules ("Small" and "Normal") were measured and tested by the non-parametric Mann-Whitney-U-test. A non-parametric test of the Spearman correlation coefficient was performed to determine whether a significant relationship exists between these two types of capsules ("Small" and "Normal") and the temperature and sunshine data collected for the days t = +5, +4, +3, +2, +1, 0, -1, -2, -3, -4 and -5, with t = 0 corresponding to the crossing day.

Relationships between embryo size, seed size and seed viability

During the elaboration of paper III, a test of seed viability using Tetrazolium was conducted to determine whether differences in viability exist between the seeds produced from German introduced populations and the seeds collected in the native area of *V. filiformis* (Georgia). The protocol and the sampling of this test are described in paper III. During the seed dissection, the length of the embryos was measured under a graduated binocular as well as the seed size. The parametric test of Pearson product moment´s correlation coefficient was performed to observe the significant relationships between seed size, embryo size and seed viability determined by the Tetrazolium test (Table 1).

5.3.3 Results

Only the length of the ovary (white line on Figure 1_A) follows a non-normal distribution and presents two types of capsules defined as "Small" and "Normal" (Shapiro-Wilk test: W = 0.952, df = 49, p- value = 0.041; Figure 1_B). Other measures (maximum length, maximum width, perimeter, area) have all normal distributions (all p-value> 0.05, data not shown). The limit between the two types of capsules is the size of 3mm. When the length of the ovary is less than 3mm, then the capsule belongs to the category noted as "Small", whereas when the ovary size is larger than 3mm, it belongs to the category "Normal" (Figure 1_B). Both types of capsules have significantly different numbers of seeds (n_{small} = 5.2 / n_{normal} = 7.8; H = 8.358, p-value = 0.0038; Figure 2_A). These seeds have significantly different sizes as well. The length of seeds coming from the "Small" capsules is significantly smaller than the length of the seeds coming from the"Normal" capsules (L_{small} = 0.989/ L_{normal} = 1.135; H = 18.798, p-value < 0.0001; Figure 2_B). It is the same for the width of the seeds (l_{small} = 0.846 / l_{normal} = 0.763; H = 7.851, p-value = 0.0050; Figure 2_C). No significant relationship was found between the type of capsule and the data of temperature or the quantity of sunshine per day. However, a strong relationship between the seed size and the embryo size exists (Pearson product moment correlation coefficient's: r = 0.601, p-value < 0.0001; Table 1 & Figure 3). This relationship varies depending on the origin of the studied seeds (Figure 4_A). Indeed, the seeds produced by the introduced populations present a stronger relationship between their sizes and the size of their embryos (r = 0.775, p-value < 0.0001; Figure 4_B) than the seeds collected in the native area (r = 0.502, p-value < 0.0001; Figure 4_B). This difference is highly significant (ANCOVA: F = 15.67, p-value < 0.0001). In addition, a strong relationship exists between embryo size and seed viability tested by tetrazolium (Pearson: r = 0.215, p-value <

0.0001; Table 1). It is important to note that the relationship between the seed size and seed viability is not significant (Pearson: r = 0.099, p-value > 0.05, Table 1).

5.3.4 Conclusions

V. filiformis presents a clear and strong variation in capsule size related to variations in seed number and size. This capsule variation has previously been observed by Lehmann (1909) in the native area of the species. This variation in capsule size present in *V. filiformis* does not appear to be associated with temperature or quantity of sunshine per day, although controlled-environment experiments should be investigated to confirm or not these hypotheses. However, the seed viability of *V. filiformis* (obtained by tetrazolium test) is not related to the seed size. The seed viability depends to the embryo size and not to the seed size.

5.3.5 Contents

- **Figure legends**

Fig. 1. Variation of capsule size in *V. filiformis*

A) example of variation in capsule size.

The two capsules come from the same individual (mother-plant "KT6" from the Kirchentellinsfurt population) after two different crossings (one done the 14th April with the pollen of the individual "TB1" of the Tübingen botanical garden and the other one done the 16th April with the pollen of the individual "Bu10" of the Burlafingen population, close to Ulm). The picture was taken the 6th May, so 22 days after the first crossing and 24 days after the second.

B) distribution of the ovary size (see white line in Figure 1_A) among the capsules produced during the crossing experiment.

Two different groups of capsule were found: the "Small" one under 3mm and the "Normal" one up to 3mm of long. The Shapiro-Wilk test indicates clearly that these two groups exist (p-value = 0.041).

Fig. 2. Number of seed per capsule (A) as well as length (B) and width (C) of the seed size in function of the type of capsule

The two pictures on the left side represent the two possible types of capsule: "Normal" and "Small". The number of seeds per capsule as well as the size of these seeds (length and width) were measured and compared between the two types of capsule. A non-parametrical Mann-Whitney-U-test indicate that the type "Small" of capsule has significantly less seeds inside than the

type “Normal” and the seeds from the “Small” type are significantly smaller in length and width than the “Normal” one: the significance levels are * = P ≤ 0.05, ** = P ≤ 0.01 and *** = P ≤ 0.001.

Fig. 3. Relationship between seed and embryo sizes

A) the relationship is represented here in function of the Georgian regions and the German populations where the seeds come from.

The parametrical Pearson product-moment correlation coefficient was tested using SPSS with the following significance levels: * = P ≤ 0.05, ** = P ≤ 0.01 and *** = P ≤ 0.001.

B) the relationship is represented here in function of the areas where the seeds come from: Native area on the left side (combining the Georgians regions) or Introduced area on the right side (combining the German populations).

Two parametrical Pearson product-moment correlation coefficients were tested using SPSS with the following significance levels: * = P ≤ 0.05, ** = P ≤ 0.01 and *** = P ≤ 0.001.

The analysis of covariance (ANCOVA) was used to compare the two Pearson product-moment correlation coefficients of the both areas together and determine if they are different. The analysis of covariance (ANCOVA) was tested using SPSS with the following significance levels: * = P ≤ 0.05, ** = P ≤ 0.01 and *** = P ≤ 0.001.

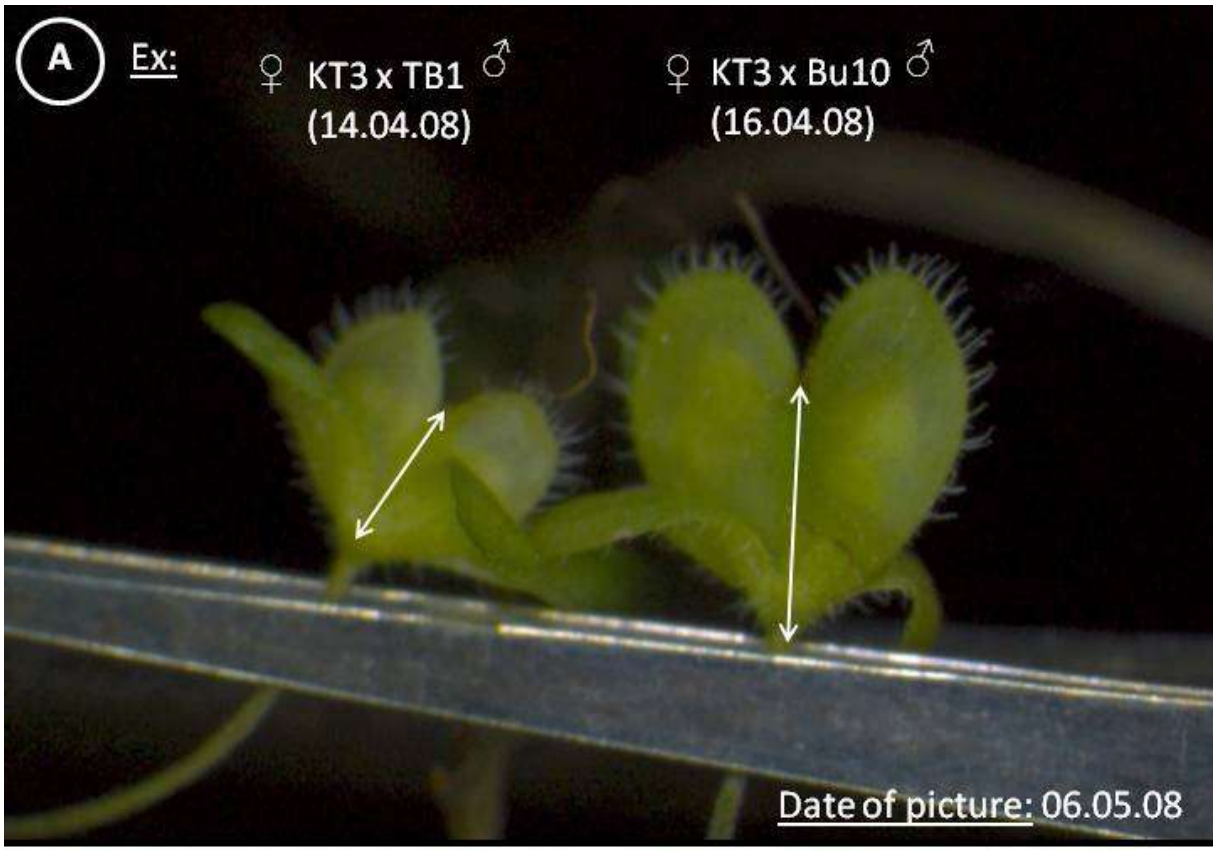
ANCOVA: F = 15.67 *** (P-value < 0.001)

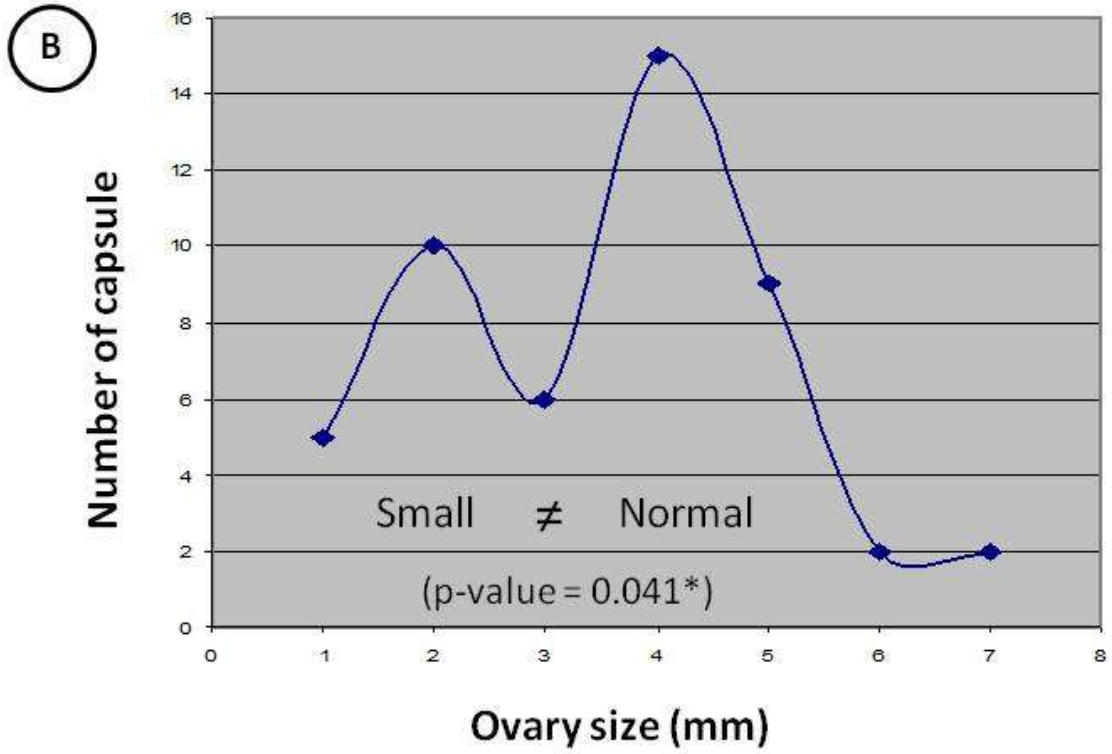
- **Table legends**

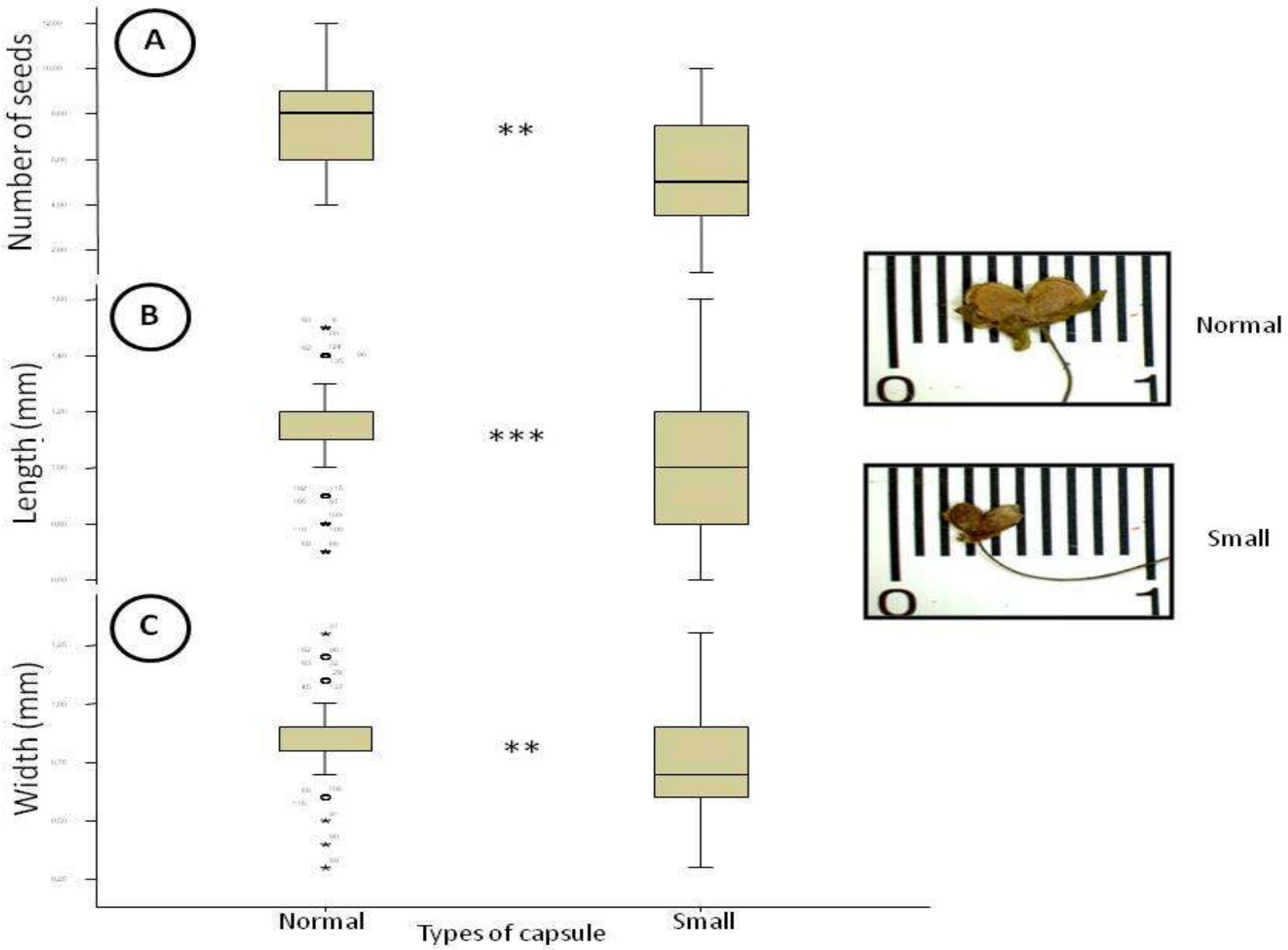
Table 1. Relationships between embryo size, seed size and seed viability

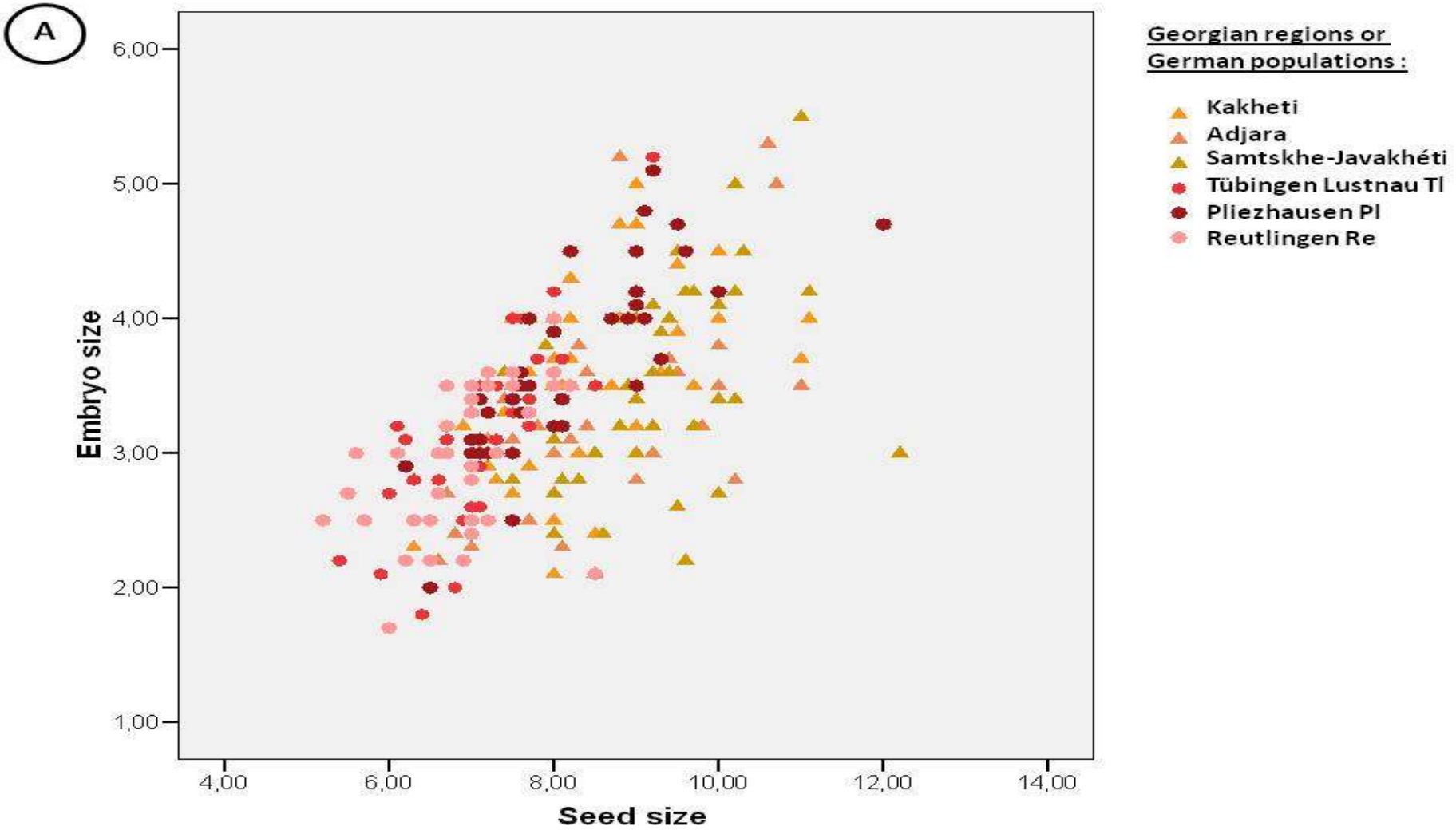
The seed viability was tested using the Tetrazolium test and the seed and embryo sizes (in mm) were measured during the test by a graduated microscope.

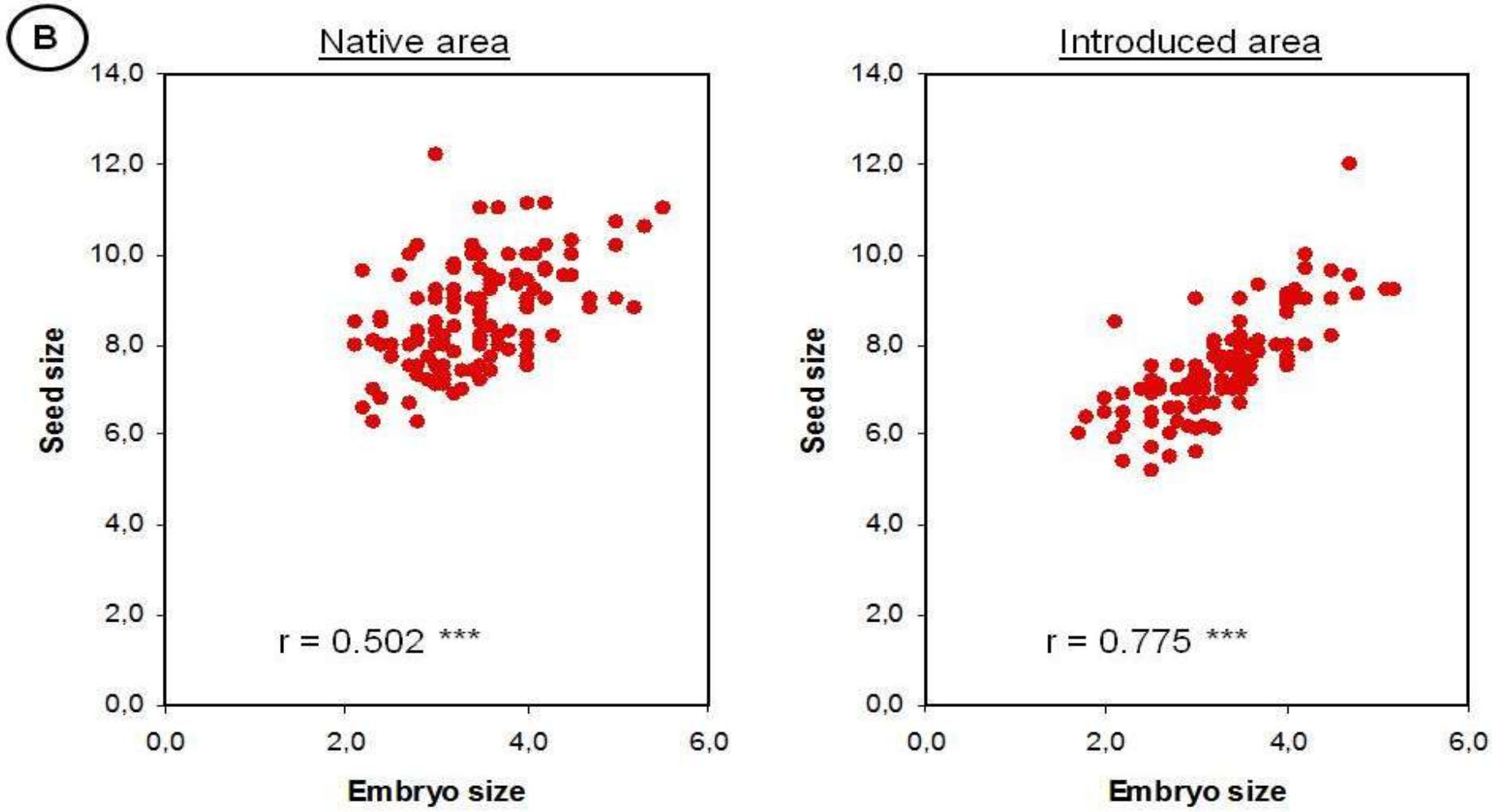
The parametrical Pearson product-moment correlation coefficient was tested using SPSS with the following significance levels: * = P ≤ 0.05, ** = P ≤ 0.01 and *** = P ≤ 0.001.


A
Ex:
♀ KT3 x TB1 ♂
(14.04.08)
♀ KT3 x Bu10 ♂
(16.04.08)
Date of picture: 06.05.08


B
Number of capsule
Ovary size (mm)
Small ≠ Normal
(p-value = 0.041*)
0
2
4
6
8
10
12
14
16
0
1
2
3
4
5
6
7
8

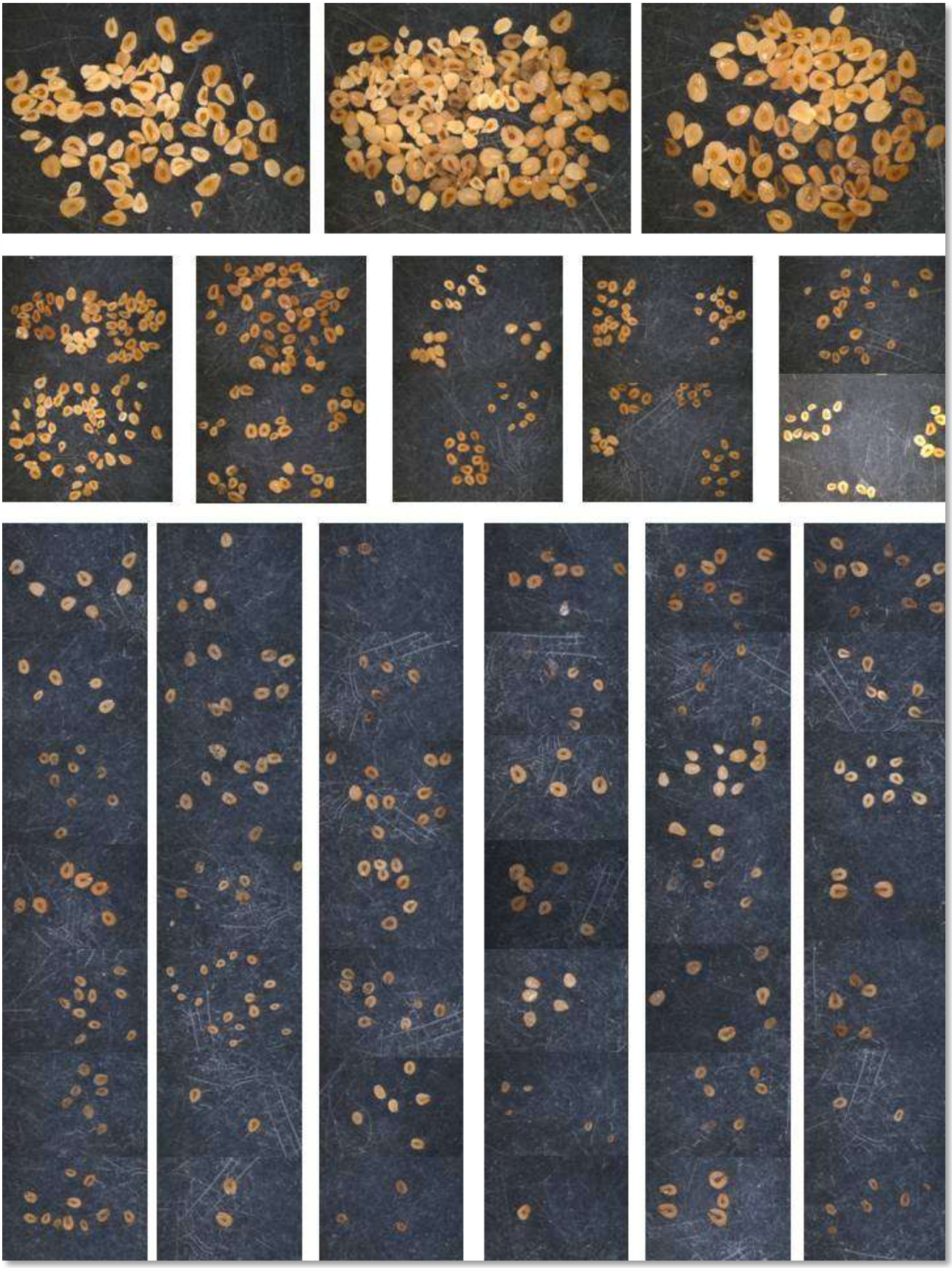

A
B
C
Number of seeds
Length (mm)
Width (mm)
**
***
**
Normal
Small
Types of capsule
Normal
Small


A
6,00
5,00
4,00
3,00
2,00
1,00
Embryo size
4,00
6,00
8,00
10,00
12,00
14,00
Seed size
Georgian regions or
German populations :
Kakheti
Adjara
Samtskhe-Javakhéti
Tübingen Lustnau Tl
Pliezhausen Pl
Reutlingen Re


B
Native area
Introduced area
Seed size
Embryo size
14,0
12,0
10,0
8,0
6,0
4,0
2,0
0,0
r = 0.502 ***
r = 0.775 ***

	Seed viability (1)	Seed size (2)	Embryo size (3)
(1)	1.000		
(2)	0.099	1.000	
(3)	0.215 ***	0.601 ***	1.000

“DIVERSITY OF SEED SIZE & NUMBER IN *V. FILIFORMIS*”



6 ABSTRACTS

6.1 ABSTRACT OF PAPER ONE

The pollen-ovule ratio (P-O) is commonly used to estimate the mode of sexual reproduction and pollination efficiency in flowering plants. In previous studies, a clear correspondence has been detected between this character and the degree of autogamy. We here investigate the evolution of this character and its putative correlates in the genus *Veronica* (Plantaginaceae). Pollen-ovule ratios of 45 species belonging to nine different subgenera and representing eleven per cent of all the species of *Veronica* were investigated and compared with results of crossing experiments from previous studies. In addition multiple populations of 17 of the 45 studied species were sampled and a controlled-environment experiment was conducted to evaluate the extent of intraspecific variation. Moreover, relationships between P-O and other primary and secondary reproductive characters of the *Veronica* flower were investigated in relation to a phylogenetic hypothesis. Our intraspecific investigations demonstrate that the P-O can vary greatly as a function of population or species history but also depends on the environment and ploidy level. Nevertheless, the differences in P-O between species correspond well to the diversity of sexual reproductive systems in *Veronica* and are correlated well with other floral characters such as stamina-style ratio or corolla size. As a consequence, a certain syndrome can be inferred for the genus. Corolla size, stamen-style ratio and P-O together seem to allow a powerful, cheap and fast tool to infer modes of plant sexual reproduction. However, causes for intraspecific variation of P-O, such as overlooked intraspecific structure or different growth conditions, need to be considered.

6.2 ABSTRACT OF PAPER TWO

The Balkan Peninsula is considered the most important refugium for species during the Pleistocene glaciations and today harbours c. 2000 endemic species, but we know surprisingly little about the evolution of taxa in this region. *Veronica saturejoides*, *V. thessalica* and *V. erinoides* are a group of closely related alpine taxa endemic to the Balkan Peninsula. Here, we analyse four DNA regions [the nuclear chalcone synthase intron (CHSi) and ribosomal internal transcribed spacer (ITS) region and the plastid r*poB-trnC* spacer and *trnL-trnL-trnF* region] and amplified fragment length polymorphism (AFLP) fingerprints to provide a phylogenetic hypothesis for the relationships among these taxa. Additionally, we analyse leaf morphological characters used to distinguish the three subspecies of *V. saturejoides*. The analyses support the distinction of the three subspecies based on previously intuitively suggested characters. Nuclear chalcone synthase intron data indicate that the southern taxa are

genetically much more diverse than the more northern *V. saturejoides* subsp. *saturejoides*. Phylogenetic relationships inferred from this region and AFLP fingerprints support the monophyly of *V. saturejoides*. In contrast, plastid DNA regions suggest a closer relationship of *V. saturejoides* subsp. *saturejoides* to *V. thessalica*. The most likely scenario involves introgression into *V. saturejoides* subsp. *saturejoides* from *V. thessalica.*

6.3 ABSTRACT OF PAPER THREE

The Baker´s law predicts that self-incompatible plant species are poor colonizers because their mating system requires a high diversity of genetically different individuals. However, the case of the invasion of the obligate gametophytic self-incompatible *Veronica filiformis* (Plantaginaceae) constitutes an exception to this rule. The species was introduced from subalpine areas of the Pontic-Caucasian Mountains and invaded many parts of Central and Western Europe during the last century (since 1893) without seed production. Intra- and inter-population crossings on nineteen introduced German populations were performed in the greenhouse confirming that introduced populations are sterile in intra-population crossings but fertile at the inter-population level. To investigate the consequences of the absence of sexual reproduction in this obligate outcrosser since its introduction, AFLP fingerprints, flower number and morphology, pollen and ovule production together with seed investigations were conducted on introduced and native populations. The genetic results confirm the presence of bottlenecks and dispersal of clones. Flower morphology revealed the frequent presence of deleterious mutations affecting the androecium of the flower and decreasing pollen production within introduced populations. The seeds produced in our transplantation and crossing experiments are smaller, have a lower germination rate and have more aborted embryos than the native individuals. Altogether, the study demonstrates that the lack of sexual reproduction led to a decrease in fitness and an accumulation of phenotypically observable mutations in reproductive characters most likely as a consequence of the accumulation of deleterious mutations via Müller´s ratchet.

6.4 SUMMARY

The sexual reproduction is a universal trait permitting to create genetic variation within flowering plants. The evolution of sexual reproductive systems has been here studied on several levels of time, in different types of habitat and using almost all the possible methods present in laboratory, in greenhouse as well as into the field. Three main parts are present in this thesis and correspond to three different papers each one with a different level of time:

genus, subgenus and species. The first part proves that the P-O ratio investigations must be systematically investigated in each plant genus or subgenus. This cheap, fast and powerful tool can produce a lot of information on the modes of plant reproduction without the using of expensive and long crossing experiments. Moreover, this measure could also give complementary data on the taxonomy of these investigated groups. The second paper treats more about the taxonomy of *Veronica* species than the two others and reveals that some events of interspecific reproduction can occur in a region of hotspot of the European biodiversity (Balkan Peninsula). These events make the morphological and genetic data incongruent and the analysis of the taxonomy of these species or subspecies difficult. The last paper narrates the story of one successful invasion occurring during the last century in Europe in despite to the fact that the species is obligate self-incompatible and that no seed observation were done in the introduced range. This paper explains the way used by the plant to circumvent the Baker´s rule (predicting unsuccessful invasion for self-incompatible species), to invade a large part of the European turfs and presents the genetic and morphological consequences of this diverging way.

Finally these three different papers try to clarify the link between the micro- and the macroevolution of the sexual reproduction in *Veronica* while discussing the relations of sexual systems with taxonomy, phylogeny, migration or environment.

6.5 ZUSAMMENFASSUNG

Die geschlechtliche Fortpflanzung ist ein universelles Merkmal und erlaubt es genetische Variation innerhalb von Blütenpflanzen zu schaffen. Die Evolution der sexuellen und reproduktiven Systeme wurde hier auf mehreren zeitlichen Ebenen, in verschiedenen Arten von Lebensraum studiert und mit fast allen möglichen Methoden im Labor, im Gewächshaus sowie im Feld untersucht. Drei Hauptteile sind in dieser Arbeit enthalten und entsprechen jeweils einem unterschiedlichen Niveau der Zeit: Gattung, Untergattung und Arten. Der erste Teil zeigt, dass die PO-Verhältnisse Untersuchungen systematisch in jeder Pflanzen-Gattung oder Untergattung untersucht werden müssen. Dieses güngstige, schnelle und leistungsstarke Werkzeug kann eine Vielzahl von Informationen über die Modi der Pflanzenreproduktion produzieren, ohne die Verwendung von teuren und langen Experimenten. Darüber hinaus könnte diese Maßnahme auch ergänzende Daten über die Taxonomie dieser untersuchten Gruppen geben. Das zweite Kapitel befasst sich mehr mit der Taxonomie der Ehrenpreis(Veronica)-Arten als die beiden anderen und zeigt, dass verschiedene Ereignisse der interspezifischen Reproduktion in einem der Hotspots der Artenvielfalt in Europa (der

Balkan-Halbinsel) auftreten. Die Ergebnisse zeigen, dass morphologische und genetische Daten inkongruent sind und die Analyse der Taxonomie dieser Arten oder Unterart schwierig ist. Das letzte Kapitel erzählt die Geschichte einer erfolgreichen Invasion, die während des letzten Jahrhunderts in Europa ablief trotz der Tatsache, dass die Arten obligate Fremdbefruchter sind und dass keine Samen-Produktion in der Region beobachtet wurde. Dieses Manuskript erläutert den Weg der Pflanze, um die "Baker-Regel" zu umgehen. Diese Regel besagt, dass selbst-inkompatible Arten erfolgloser bei der Invasion neuer Lebensräume sind. Dennoch schafft es die hier untersuchte Art einen großen Teil der europäischen Rasen zu bevölkern und zeigt dabei genetische und morphologische Veränderungen auf diesem Weg.
Schließlich wird in diesen drei verschiedenen Papieren versucht, die Verbindung zwischen der Mikro-und Makroevolution in der geschlechtlichen Fortpflanzun in Ehrenpreis (Veronica) unter Betracht verschiedener sexueller Systeme und der Stammesgeschichte, sowie der Migration zu klären.